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# SEARCH FOR NEW ANTIBIOTICS AGAINST RESISTANT STAPHYLOCOCCI

The report of discovery of new antibiotics at present time is not heralded with the same enthusiasm that was evinced in the early days of the antibiotic era some two decades ago. Any new development has to be evaluated against vast data already accumulated. In the search for new antibiotics, no other organism has played a more important part than *Staphylococcus aureus* (= *Micrococcus pyogenes* var. *aureus*) popularly called by medical people as "Staph." It is so widespread, inciting a variety of disease symptoms, that the discovery of an effective antistaphylococcal antibiotic offers a good commercial proposition to the pharmaceutical industry. Since the starting of antibiotic therapy for infectious diseases, the staphylococci have become very important from point of view of public health. Strains of staphylococci resistant to the life-saving antibiotics such as penicillin, streptomycin, tetracyclines and chloramphenicol are so easily developed, that a new menace has to be faced.

It may be of interest to note that before 1946, there were hardly any indications of penicillin resistant staphylococci in Boston City Hospital. In 1952, Spink pointed out that in the same hospital where large amounts of penicillin were being used, 25 per cent of the staphylococci isolated from patients were penicillin resistant. By 1954 more than 50 per cent of the strains were found to be penicillin resistant. The antibiotic had less effect in curing the disease and this resulted in higher mortality. Hospital acquired staphylococcal infections, cross-infection of different types between patients, presence of symptomless carriers among the hospital personnel have all contributed to the establishment and spread of antibiotic resistant and virulent strains of staphylococci among the people, thus presenting a grave public health problem. This has also kept the research workers looking for new antibiotics which would be effective against the resistant staphylococci.

As far back as 1944, Kirby reported the occurrence of penicillin resistant staphylococci, the organism producing an inactivator of penicillin. Staphylococci belonging to the phage types 80/81, with

positive coagulase, hemolysin, gelatin liquification and mannitol fermentation have been shown to be highly virulent and capable of developing resistance to the commonly used antibiotics. Novobiocin produced by *Streptomyces sphaeroides* was the first antibiotic to be introduced as being effective against resistant staphylococci and pneumococci. It has no cross resistance with any other antibiotic used clinically at present. Even though resistant staphylococcal strains are not readily developed, the antibiotic is found to be highly bound to plasma protein, so that, effective blood levels are not easily obtained.

Oleandomycin produced by *Streptomyces antibioticus* has been used in combination with penicillin and tetracycline to suppress the emergence of antibiotic resistant staphylococci. Erythromycin is the other antibiotic used for the same purpose, and according to Garrod it is more effective than oleandomycin. A triacetyl derivative of oleandomycin is also used clinically. Spiramycin produced by *S. ambofaciens* is similar to erythromycin, and shows some cross-resistance with it.

Two new antibiotics against which staphylococci do not develop resistance readily, were discovered recently and deserve special mention : ristocetins A and B produced by *Nocardia lurida* and vancomycin produced by *S. orientalis*. The latter organism has been isolated by Eli Lilly research workers from soil samples collected in India. Staphylococci do not readily develop resistance against these two antibiotics. However, the antibiotics have to be administered to the patients by intravenous route, so that they are used only in acute cases of infection where other antibiotics have failed. Kanamycin produced by *S. kanamyceticus* reported by Umezawa *et al.*, from Japan, is also effective against resistant staph, but like streptomycin, has side reactions inciting auditory disturbances. The search for new antibiotics against resistant staphylococci goes on, with the hope, that some day, an effective antibiotic without any undesirable effects would be discovered.

## REVIEW

# Refrigeration and Air-Conditioning for the Pharmaceutical Industry

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REFRIGERATION and air-conditioning are of considerable importance to a large number of industries, but particularly so in the pharmaceutical industry. A pharmaceutical factory requires large supplies of conditioned air, and besides temperature and humidity, other conditions of the air also require to be controlled.

The subjects refrigeration and air-conditioning are often grouped together as air-conditioning mostly involves refrigeration of air either to bring down its temperature or to reduce the humidity.

### Refrigeration

Broadly speaking there are two methods of achieving refrigeration on a commercial scale :

(a) Change of phase; and
(b) Joule-Thomson Effect.

The change of phase method can be subdivided further into two distinct methods: (1) compression, and (2) absorption.

The change of phase method is the more commonly used as the refrigeration quantum produced by Joule-Thomson Effect is quite small. Joule-Thomson Effect is mostly employed for regenerative cooling in liquification of air. Under change of phase method, the compression method is more common than absorption.

Among the types of compressors used, reciprocating compressors have been popular in the past, but more recently for large capacities and comparatively small compression ratios centrifugal machines are finding favour. Steam jet refrigeration is classified under compression methods and finds limited application where the range of cooling desired is rather small. It has particular advantages for this type of work as there are no moving parts involved. Where power is generated from steam the latter is also a cheaper source of energy and counterbalances to some extent the low overall efficiency of steam jet compression.

Refrigeration system is understood fairly easily if it is considered as a heat pump. As a centrifugal pump lifting a liquid, the capacity should be specified in terms of two different parameters. In case of centrifugal pumps, these parameters are (a) quantity of liquid per unit time such as g. p. m., and (b) total dynamic head, such as foot. For a refrigeration system, the quantity of heat to be removed in unit time as well as the temperature at which this heat is to be removed should be specified. The lower the temperature at which the heat is to be removed the higher will be the power requirements of the system. Unfortunately the unit selected for expressing the capacity of a refrigeration system is not specific and may often prove confusing. For example, in the United States the most commonly used unit is 1 ton of refrigeration and this expression includes the element of time. The temperature conditions are also specified. The unit originated as the quantity of refrigeration required for manufacture of 1 ton of ice in 24 hours starting with water at ice temperature. The U. S. ton of refrigeration today is defined as extraction of 288,000 BTU per 24 hours at a suction

pressure which corresponds to a saturation temperature of 5°F and a delivery pressure corresponding to 86°F. The British system is a little more scientific and has adopted 1 kg. calorie per second, which amounts to 342,860 BTU per 24 hours, as the unit of refrigeration with the standard conditions of a temperature range of cooling water from 15°C at inlet to 20°C at outlet.

The refrigerating capacity of a machine when expressed as tons of refrigeration under the U. S. system is indeed very different from the actual ice making capacity of a plant. The ice capacity is about half of the refrigeration capacity as there are various other refrigeration loads when ice is produced. When considering the efficiency of a refrigerating system, the term coefficient of performance is the ratio of refrigeration produced to work done by compressor. For isothermal compression, refrigeration produced is heat content of vapour leaving the evaporator less the heat content of liquid entering the evaporator, while work done by the compressor is heat content of vapour leaving the compressor less the heat content of vapour leaving the evaporator. Table I gives a comparative rating of various refrigerants commonly used in industry.

Another important criterion for efficiency of refrigerating machines is the volumetric efficiency of the compressor. Volumetric efficiency of a compressor depends to a large extent upon (a) the details of construction of the machine, (b) compression ratio, and (c) method of operation. Normally, larger the machine better the volumetric efficiency as the ratio of dead space can be made small compared to the volume swept per stroke. It is also evident that higher the compression ratio lower would be the efficiency as even a small dead space will contribute a large quantity of fluid at the suction conditions if the compression ratio is large. As regards the method of operation, in some machines the liquid refrigerant is allowed to enter the compressor in order to achieve isothermal compression. This practice is not common but wherever adopted it would require larger clearances in the compressor design and the volumetric efficiency will be lower. It should be noted that lower volumetric efficiency does not result in a proportionately higher power consumption as most of the power put in to compress the fluid is recovered when the fluid in the dead space expands again. A low volumetric efficiency, however, results in a

Table I. Comparison of refrigerants.

One-Ton Refrigeration, 5° to 86°F

| Cycle | Weight lb/min. | Volume cu. ft./min. | Ratio of compression | Coefficient of performance | Horse power/ton. | Relative efficiencies |
|---|---|---|---|---|---|---|
| Ideal .. .. .. | | | | 5.74 | 0.8214 | 100 |
| Ammonia .. .. | 0.0214 | 3.44 | 4.93 | 4.85 | 0.973 | 84.5 |
| Propane .. .. | 1.396 | 3.35 | 3.64 | 4.88 | 0.9668 | 85.0 |
| Carbondioxide .. | 3.74 | 0.999 | 3.11 | 2.56 | 1.843 | 44.6 |
| Sulfurdioxide .. | 1.388 | 9.24 | 5.63 | 4.735 | 0.995 | 82.5 |
| Ethyl ether .. .. | 1.555 | 60.8 | 7.12 | 4.86 | 0.971 | 84.6 |
| Dichlorethylene .. | 1.768 | 108.4 | 8.23 | 5.24 | 0.918 | 89.4 |
| Trichloroethylene .. | 2.137 | 513.0 | 10.84 | 5.085 | 0.928 | 88.5 |
| Water .. .. .. | 0.1996 | 1972.0 | 21.9 | 4.1 | 1.15 | 71.5 |

larger size compressor and thus may lead to slight mechanical inefficiency.

A close study of the simple refrigeration cycle consisting of a compressor, a condenser, a throttle valve and an evaporator reveals interesting features. Considered as a heat pump it can not only give us refrigeration during summer but the same machine can be used for giving heat to enclosed spaces during winter. As the coefficient of performance for most systems is much higher than one, the efficiency of refrigerating systems when used as heater is much higher than direct electrical heating. When these machines are used as heater, the condenser is located in the enclosed space to be heated and the evaporator uses water from deep wells for evaporation of the refrigerant. It normally happens in cold countries that water from deep wells has a constant temperature almost throughout the year. Such use of refrigerating machines is not uncommon in western countries where they have severe winters. As a matter of fact the refrigeration machines work on a reverse cycle for most part of the year and work as refrigerating machines only for one or two short summer months. In the northern latitudes of India also there are unit conditioners which work on this principle.

**Air-Conditioning**

Complete air-conditioning should include control of the following properties of air:

(i) Temperature;

(ii) humidity;

(iii) movement and circulation;

(iv) cleanliness, freedom from dust, fumes, smoke etc.;

(v) sterility, *i.e.*, freedom from bacteria, moulds etc.; and

(vi) positive pressure inside the room.

For control of temperature, air is usually passed outside finned tube bundles carrying chilled water. Some times if the temperature desired is not very low, air is passed through water sprays to achieve evaporative cooling.

Humidity is controlled by one of the two methods:

(a) Chilling the air to a very low temperature; and

(b) chemical dehydration.

The most commonly used chemical dehydrants are:

(a) Silica gel;

(b) activated alumina;

(c) lithium chloride;

(d) triethylene glycol; and

(e) calcium chloride.

Usually the dehydrators are kept in compartments and regenerated in cycles for use over and over again.

The movement and circulation of air is of considerable importance. Very often even under comfort conditions of temperature and humidity people inside an air-conditioned room may feel suffocated if the quantity of air and its circulation are not properly regulated. Direct drafts should be avoided but at the same time there should be reasonable velocity of air in all parts of the room. About 6 to 10 changes of air per hour are considered to be normal.

The cleaning and purification of air is achieved by mechanical filters or where essential by electrostatic precipitators. Centrifugal collectors are also used as primary devices for removal of larger particles of foreign materials from air.

More recently activated carbon filters have found much favour even in preference to electrostatic precipitators. A major part of sterilisation is achieved if the air is cleaned and purified by these means. More rigorous sterilisation is usually accomplished by ultra violet irradiation at 253.7 m$\mu$. For irradiation of air to be sterilised through a special irradiation chamber the U. V. lamp capacity should be about 20 watts of radiation per 1000 c.f. m. air. The relative humidity of the irradiated air should be maintained at 50 per cent or below for maximum bactericidal effect.

As regards requirements of positive pressure inside sterile rooms, a value of about 0.1″ water gauge is recommended though very often much higher values are used in certain installations. Such pressure should be measured by an inclined tube manometer. This pressure requirement in a sterile room is of very great importance and some times presents difficult problems involving not only the air-conditioning plant but the layout of the ancillary rooms as well. Experience has shown that conventional air-conditioning plants having separate air inlet and extract fans cannot be relied upon completely to maintain a positive pressure within the sterile area even when fans of a larger inlet capacity than the extract ones are allowed. Variation in filter resistance, fan belt slippage, wind effect, etc., may influence the performance of the inlet system and some times reduce the designed level of positive pressure to such an extent that it may be even negative.

While for comfort conditions 6 to 10 changes per hour may be considered sufficient, more frequent changes may be required under certain conditions, as in rooms having high heat loads due to equipment or the number of persons inside the enclosed space. The heat capacity of air is rather small and 100 cu. ft. of air can take only about 3 BTU for 1°C rise. Again, the number of air changes required from point of view of bacterial decontamination is also greater than the lower limit for comfort conditions. As many as 20 changes per hour are recommended for sterile areas. This can be expressed also as a mathematical relationship, $R = \frac{138}{t} (\log_{10} n_1 - \log_{10} n_2)$, where R is the number of air changes per hour, t the time in minutes for reduction of number of infected particles from $n_1$ to $n_2$. This equation is based on the simple logarithmic law for rate of disappearance of bacteria at any moment which is proportional to the bacterial concentration at that moment. One important term commonly used in air-conditioning parlance is comfort zone or comfort conditions. The temperature and humidity at which any person feels comfortable vary with and depend on:

(a) Individual factors, and vary from individual to individual;

(b) air movement and air distribution;

(c) outside climate; and

(d) the variations from country to country, apart from individual variations.

Certain comfort zones are designated taking into account the common reaction of the largest number of individuals. For western countries the winter comfort conditions are about 74°F and 40 per cent relative humidity while in summer it is about 80°F and 50 per cent relative humidity. These conditions refer to the centre of the comfort zone and a lower humidity permits a little higher temperature and vice-versa for comfort.

While the above refer to human comfort, the industry requires different conditions for best results. Table II and comments following give conditions required for different pharmaceutical operations.

TABLE II.

TEMPERATURE AND HUMIDITY CONDITIONS FOR PHARMACEUTICAL INDUSTRY

| | Temp. °F | RH % |
|---|---|---|
| Powder storage (prior to mfg.) | 70 to 80 | 30 to 35 |
| Manufactured powder storage and packing areas | 75 to 80 | 15 to 35 |
| Milling room | 80 | 35 |
| Tablet compressing | 70 to 80 | 40 |
| Tablet coating room | 80 | 35 |
| Effervescent tablets and powders | 90 | 15 |
| Hypodermic tablets | 75 to 80 | 30 |
| Colloids | 70 | 30 to 50 |
| Cough syrups | 80 | 40 |
| Glandular products | 78 to 80 | 5 to 10 |
| Ampule manufacturing | 80 | 35 |
| Gelatin capsules | 78 | 40 to 50 |
| Capsule storage | 75 | 35 to 40 |
| Micro-analysis | 80 | 50 |
| Biologicals manufacturing | 80 | 35 |
| Liver extracts | 70 to 80 | 20 to 30 |
| Sera | 74 to 78 | 50 |
| Animal rooms | 75 to 80 | 40 |
| Small animal rooms | 75 to 78 | 47 to 48 |

Gelatin capsules require varying relative humidities, depending upon size of capsule. Moisture content should not exceed 0.25 g. per cu. ft. Various kinds of gelatin require different temperatures.

Penicillin incubation process requires holding temperature within 0.5°F, with temperature and humidity rigidly controlled during all manufacturing phases.

Ampule filling requires a 20 per cent relative humidity when especially fine powders are handled.

Uncoated tablet manufacturing requires accurate control of temperature and relative humidity, since low relative humidity causes formation of a hard outer layer, and high relative humidity retards drying at the proper rate.

Liver extracts require a low relative humidity after they are dried. Temperatures higher than 80°F will cause the extracts to deteriorate.

Tablet coating requires the control of the temperatures of all ingredients and the temperature of air introduced into coating pans.

Sterile conditions are essential in many pharmaceutical processes. Suitable air exhaust to remove surplus material from tablet compressing machine should be provided.

Air filtration is generally required, with positive air filtration in some areas.

In the design of a proper air conditioning system a number of factors have to be considered in order to achieve the desired results. The most important of these factors are summarized below:

A. Temperature and/or humidity to be maintained.
   1. Allowable seasonal variations.
   2. Degree of acuracy required.

B. Outdoor conditions to be assumed for design (not necessarily the extremes encountered).

C. Architectural plans and details of building construction. If original plans are not available, the building must be carefully measured and details of construction determined by inspection.
   1. Orientation of building with regard to the sun.

2. Zoning requirements due to sun effect, load concentrations, and differences in conditions required for various processes.

D. Sensible heat gains.
1. Solar radiation and transmission through glass.
   (a) Kind of glass.
   (b) Shading devices.
2. Transmission, including sun effect, through walls and roofs.
3. Power.
   (a) Usage factor.
   (b) Per cent loaded.
4. Lighting.
   (a) Usage factor.
   (b) Auxiliaries.
5. Miscellaneous: ovens, exposed steam pipes, etc.
6. Product entering at above space temperature.

E. Latent heat gains.
1. Free water used in process.
2. Seepage of water vapour through building materials (especially important in low dew-point applications).
3. Water vapour from moist product.

F. Sensible and latent heat gains.
1. People.
   (a) Degree of activity.
   (b) Duration of occupancy.
2. Gas-burning equipment.
   (a) Usage factor.
   (b) BTU value of gas used.
3. Heating equipment evaporating water (capacity and usage factor).
4. Chemical and biological reactions.
5. Infiltration of air.
   (a) Frequency of door openings.
   (b) Window cracks.
   (c) Porosity of building structure.

G. Ventilation air.
1. For human occupancy.
   (a) Per cent smoking.
2. Toxic fume and smoke dilution.
3. Odour dilution.
4. Offsetting exhaust hood requirements.

The design of a proper air-conditioning system is a job for a specialist but its proper maintenance and analysis of its working is an interesting job for a chemical engineer.

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## ORIGINAL PAPERS

# Studies on Penicillinase

### I. A MODIFIED RAPID IODOMETRIC METHOD OF ASSAY

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VARIOUS methods of penicillinase assay have been described in literature. The cup-plate bioassay method[1] of determining residual penicillin after penicillinase action and also the iodometric method of Perret[2] are time consuming. The manometric method,[3] although gives accurate results, is expensive and limited by the availability of equipment, and therefore not quite suited for routine analysis of large number of samples.

The iodometric method of assay is based on the enzymatic decomposition of penicillin to penicilloic acid which is known to react stoichiometrically with iodine to give a compound which produces no colour with starch. Pollock[4] first indicated that a rapid approximate assay of penicillinase could be done by measuring the time required to decolorize a known quantity of iodine in presence of sufficient substrate. More recently Citri,[5] and Citri and Garber[6] described an iodometric assay method on the lines suggested by Pollock.[7] We have made a detailed study of this problem and standardized a two-minute assay method with suitable modifications. Penicillinase assay values by this method agree very well with those obtained by the manometric method.[3] This quick and simple method, described below, has been particularly useful during the isolation and purification of penicillinase from *Bacillus cereus* fermentation broth. The details of purification procedure will be described in a subsequent publication. Some of the properties of purified penicillinase are also described in this paper.

## MATERIAL AND METHODS

### Penicillinase

The purified penicillinase preparation was made from fermentation broth obtained by fermenting *Bacillus cereus* 5/B NCTC (9946) in casein hydrolysate medium as described by Pollock[7] with the modification that the fermentation was carried out on a rotary shaker at 24-25° in two stages. This highly purified preparation of penicillinase is of an injectable grade and is designated "Antipen".** All data reported here were obtained with Antipen.

### Reagents

1. Iodine solution: 0.01*N* iodine in 0.1*M* potassium iodide.

2. Penicillin G (substrate): Crystalline sodium penicillin G (Hindustan Antibiotics Ltd.) 1660 u/mg., dissolved in 0.1*M* phosphate buffer, pH 7.0, to contain not less than 5,000 u/ml.

3. Gelatin solution: 1 per cent (c. p. grade, E. Merck) in 0.1*M* phosphate buffer, pH 7.0.

4. Penicillinase solution: Prepared by dissolving Antipen in 1 per cent

* Present address: Central Drugs Laboratory, Kyd Street, Calcutta.

** Trade name of Hindustan Antibiotics Ltd. for injectable penicillinase.

aqueous gelatin, unless otherwise stated, to contain between 120 to 360 u/ml.

5. Starch solution: 1 per cent soluble starch in water.

*Unit* : Penicillinase activity is expressed in Pollock and Torriani[8] unit. One unit is that amount of enzyme which will hydrolyse 1 $\mu$M of sodium penicillin G in one hour at pH 7.0 at 30°.

**Method of assay**

All reagents are equilibriated to 30° in a water bath before adding them to the reaction tubes (20 x 150 mm. Pyrex test tubes) placed on a rack at 30° in the following order :

1 ml. of gelatin solution
1 ml. of enzyme
1 drop of starch solution
1 ml. of penicillin solution
2 ml. of iodine.

The time of decolorization of iodine is recorded with a stop-watch from the time of addition of penicillin solution, which is blown into the reaction mixture from a 1 ml. pipette. Addition of iodine is made within 10-15 sec. after addition of substrate. A substrate blank should always be determined using water in place of enzyme solution. Freshly prepared penicillin solution will not show any blank. Blank iodine titre, if any, should be added to the 2 ml. of the iodine reagent. Penicillinase units per ml. of enzyme solution is read from Table I which shows units of enzyme against time of decolorization of 2 ml. of 0.01*N* iodine. Table I, which gives fairly accurate values, was prepared from the following considerations.

By definition, 1 unit of penicillinase hydrolyses 1 $\mu$M of sodium penicillin G, *i.e.*, 600 units of penicillin (593 units to be exact) in 1 hr. Assuming 1 ml. of 0.01*N* iodine equivalent to 600 units of penicillin (exact factor has to be determined by titrations), 120 units of penicillinase will decolorize 2 ml. of 0.01*N* iodine in 60 sec. Table I has been prepared from the observation

TABLE I.

UNITS* OF PENICILLINASE CORRESPONDING TO TIME OF DECOLORIZATION OF 2 ML. OF 0.01*N* IODINE SOLUTION

| Time in sec. | Enzyme u/ml. | Time in sec. | Enzyme u/ml. | Time in sec. | Enzyme u/ml. | Time in sec. | Enzyme u/ml. |
|---|---|---|---|---|---|---|---|
| 60 | 120 | 50 | 144 | 40 | 180 | 30 | 240 |
| 59 | 121 | 49 | 146 | 39 | 184 | 29 | 248 |
| 58 | 124 | 48 | 149 | 38 | 188 | 28 | 257 |
| 57 | 126 | 47 | 152 | 37 | 194 | 27 | 266 |
| 56 | 128 | 46 | 156 | 36 | 200 | 26 | 276 |
| 55 | 130 | 45 | 160 | 35 | 206 | 25 | 288 |
| 54 | 133 | 44 | 163 | 34 | 210 | 24 | 300 |
| 53 | 135 | 43 | 167 | 33 | 217 | 23 | 312 |
| 52 | 138 | 42 | 170 | 32 | 224 | 22 | 326 |
| 51 | 140 | 41 | 175 | 31 | 230 | 21 | 342 |
| | | | | | | 20 | 360 |

*Pollock and Torriani[8].

that within the range of 20 to 60 sec. of decolorization time, the product of enzyme activity and time of decolorization is constant. As a matter of fact with highly purified preparation of penicillinase like Antipen, this product has been found to be constant upto a decolorization time as long as 2.5 min. with lower concentrations of enzyme (Table II). The exact

TABLE II.

EFFECT OF ENZYME CONCENTRATION ON THE DECOLORIZATION TIME OF 2 ML. OF 0.01*N* IODINE

| Penicillinase activity units | Time of decolorization of iodine in sec. | Product of penicillinase activity and time of decolorization. |
|---|---|---|
| 26 | 285 | 7,410 |
| 34 | 217 | 7,378 |
| 43 | 167 | 7,181 |
| 87 | 82 | 7,134 |
| 128 | 56 | 7,168 |
| 175 | 41 | 7,175 |
| 217 | 33 | 7,161 |
| 257 | 28 | 7,196 |
| 300 | 24 | 7,200 |
| 347 | 21 | 7,237 |
| 390 | 19 | 7,410 |
| 430 | 18 | 7,740 |
| 480 | 17 | 8,160 |
| 530 | 16 | 8,480 |
| 650 | 14 | 9,100 |

unitage can be calculated by multiplying the value obtained from Table I by the factor (F) determined by actual titration of 0.01*N* iodine with penicillin as follows :

$$F = \frac{\text{Penicillin units equivalent to 1 ml. 0.01}N\text{ iodine}}{593}$$

This factor will usually vary within 1 to 1.1 depending on the purity of the penicillin standard. The enzyme unitage read from Table I will be within a maximum error of 10 per cent even if no correction is made. A rapid estimation of penicillinase within this range of accuracy can, therefore, be made within about two minutes with a 0.01*N* solution of iodine.

## RESULTS AND DISCUSSION

The method of assay described above was established as a standard procedure from the experimental observations described below.

### Effect of exposure of penicillinase to iodine

According to Pollock,[4] the exo-enzyme from *B. cereus* obtained from filtered broth is the $\alpha$-penicillinase type which was stated to be inactivated by iodine relatively slowly as compared to the cell-bound type namely $\gamma$-penicillinase which appeared to be enzymatically completely inactivated by pre-exposure to iodine. The effect of iodine exposure to our penicillinase preparation which is supposed to be of the $\alpha$-type was, therefore, studied. The enzyme activity was determined with and without the presence of gelatin in the reaction medium. For exposure to iodine, 2 ml. of 0.01*N* iodine solution was added to the enzyme solution before the addition of substrate in the standard method of assay. From the results presented in Table III, it will be seen that over 50 per cent of the enzyme activity was lost after only 30 sec. exposure in the absence of gelatin. Practically all the activity was lost after a two-minute exposure. Gelatin thus appeared to have a protective action against iodine inactivation. This protection increased with the increasing concentration of gelatin. The inactivation was only 14 per cent in presence of 1 per cent gelatin after 30 sec. exposure of the enzyme to iodine. The figures recorded under zero sec. exposure are the values obtained by standard method of assay. Since even in the absence of gelatin there was no measurable absorption of enzyme in the reaction test tube within

TABLE III.

EFFECT OF EXPOSURE OF PENICILLINASE TO 2 ML. OF 0.01*N* IODINE

| Exposure time in sec. | 0 | | 30 | | | 60 | | | 120 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Assay medium | Time for decolorization of $I_2$ in sec. | Activity u/ml. | Time for decolorization of $I_2$ in sec. | Activity u/ml. | Inactivation % | Time for decolorization of $I_2$ in sec. | Activity u/ml. | Inactivation % | Time for decolorization of $I_2$ in sec | Activity u/ml. | Inactivation % |
| Water* | 41 | 175 | 94 | 76 | 57 | 208 | 34 | 80 | 545 | 6.5 | 96 |
| 0.1% gelatin | 33 | 217 | 55 | 130 | 40 | 60 | 120 | 45 | 107 | 67 | 69 |
| 0.5% gelatin | 30 | 240 | 38 | 188 | 22 | 42 | 170 | 30 | 52 | 138 | 57 |
| 1.0% gelatin | 30 | 240 | 34 | 210 | 14 | 39 | 184 | 24 | 47 | 146 | 40 |

*Enzyme solution was made in water and the phosphate buffer did not contain gelatin.

these periods, the loss in enzyme activity can be attributed to be solely due to iodine inactivation. Contrary to the findings of Pollock,[4] it therefore appears that the extracellular penicillinase is also highly susceptible to iodine inactivation. Presumably in the presence of gelatin this inactivation was not observed by Pollock,[4] and Citri and Garber[5].

**Variation of time of iodine addition**

In the standard method which was found suitable for even crude preparations of penicillinase including unprocessed fermentation broth, the iodine solution is added immediately after initiating the enzyme reaction with penicillin substrate (within 10 to 15 sec.) With highly purified preparations it was observed that within a decolorization time period of as long as 136 sec. it made no difference whether iodine was added within 10 sec. or any time between 10 to 110 sec. after the addition of penicillin (Table IV). The accumulation

TABLE IV.

VARIATION OF TIME OF IODINE ADDITION ON PENICILLINASE ACTIVITY

| Time before $I_2$ addition in sec. | Time of decolorization in sec. | Activity of penicillinase u/ml. |
|---|---|---|
| 10 | 134 | 53.6 |
| 20 | 135 | 53.0 |
| 30 | 130 | 55.6 |
| 40 | 132 | 54.0 |
| 50 | 134 | 53.6 |
| 60 | 135 | 53.0 |
| 70 | 130 | 55.6 |
| 80 | 130 | 55.6 |
| 90 | 135 | 53.0 |
| 100 | 134 | 53.6 |
| 110 | 136 | 52.5 |

of penicilloic acid under the conditions of assay did not apparently slow down the

rate of enzyme reaction. However, with crude broth containing penicillinase, addition of iodine within 10 to 15 sec. gave reproducible enzyme titre in repeated experiments.

**Effect of change in the order of addition of reactants with increasing concentrations of iodine**

In the iodometric method described by Citri,[5] the reaction is initiated by the addition of enzyme, whereas in the method described here the substrate penicillin initiates the enzyme action. In Fig. 1 are

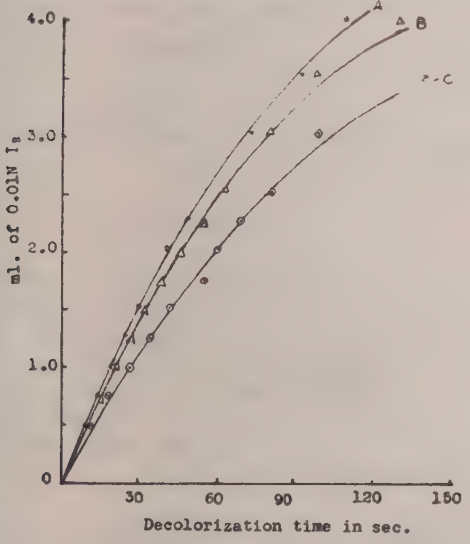


FIG. 1.

Effect of change in the order of addition of reactants on penicillinase activity with increasing conc. of $I_2$

Order of addition— Curve A, Enz + Pen + $I_2$
Curve B, $I_2$ + Pen + Enz
Curve C, Enz + $I_2$ + Pen

shown the results of experiments in which the order of the reaction mixture was varied and the time of decolorization was recorded for different amounts of iodine (0.5 to 4 ml. of 0.01*N* iodine). The final volume of the reaction mixture was kept constant at 5 ml. in all cases. The additions were made in immediate succession, *i.e.* within 10 sec. from one to the next. It will be noticed that up to 2 ml. of iodine in the reaction mixture the time of decolorization was practically identical and also linear in curve A (order of reactants as in the present method) and curve B (order of reactants as in the method of Citri[5]). With increase in the amount of iodine, curve B showed significant enzyme inactivation possibly due to simultaneous exposure of the enzyme to iodine and penicillin. In curve C the enzyme was in effect pre-exposed to iodine for about 10 sec. or so and consequently showed significant enzyme inactivation practically with all concentrations of iodine. All these experiments were carried out by our assay method in the presence of 1 per cent gelatin. The results of these experiments clearly indicate the preference of the order of addition as given in the present method of assay.

**Comparison with manometr ic method**

Penicillinase titre of Antipen solution as determined by this modified iodometric

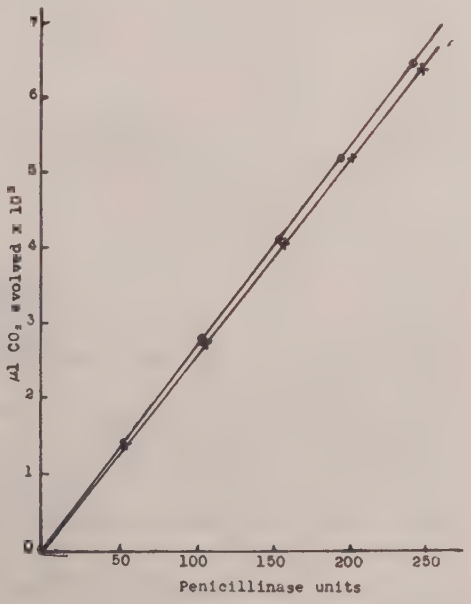


FIG. 2.

Comparison of iodometric method with manometric method

●—● Theoretical according to iodometric assay
×—× Actual

method was checked by the standard manometric method of assay[3]. From the curves shown in Fig. 2 it will be seen that there was complete agreement in the results obtained by the two methods.

### Optimum pH

The pH optimum curve of Antipen preparation is shown in Fig 3. All the experiments were done with phosphate buffers. The maximum activity was obtained at pH 7.2 which agrees very well with previous reports with *B. cereus* enzyme estimated manometrically[3]. Manson and coworkers[9] employing the iodometric method of Peneau and coworkers[10] as modified by Perret[2], however, found the optimum pH to be 6.0.

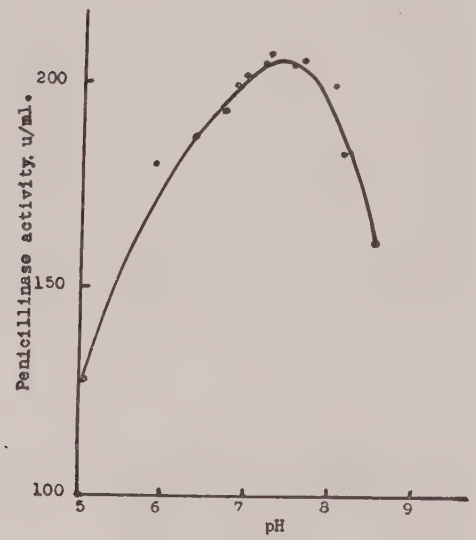


FIG. 3.

Optimum pH for penicillinase activity

### Optimum temperature

The velocity of enzyme reaction is plotted against temperature in Fig 4. The optimum temperature was found to be between 30 and 35°.

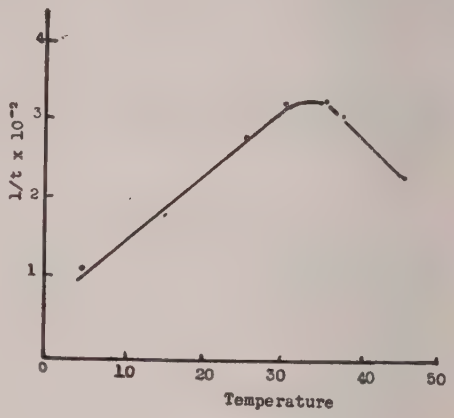


FIG. 4.

Optimum temperature for penicillinase activity. t=time of decolorization in sec.

## SUMMARY

A simple two-minute iodometric assay based on iodine decolorization time is described for accurate measurement of penicillinase activity in crude as well as purified preparations of penicillinase. For *B. cereus* penicillinase the optimum pH was found to be 7.2 and the optimum temperature 30° to 35°

Contrary to previous observations by other workers the extra-cellular penicillinase of the ∝—type from *B. cereus* was found to be highly susceptible to iodine inactivation. Gelatin showed a protective action against iodine inactivation.

## ACKNOWLEDGMENT

We are indebted to Dr. M. R. Pollock, National Institute for Medical Research, London, for supplying the strain of *B. cereus*, to Mr. G. Sen for his help in the preparation of enzyme, and to Dr. V. L. Vinze for technical assistance in manometric assay.

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# Studies on Penicillinase

## II. PURIFICATION OF PENICILLINASE

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IN a previous paper[1] a rapid iodometric method of penicillinase assay based on iodine decolorization time has been described. The development of this quick iodometric method made it possible to undertake detailed studies on the purification of this enzyme. Penicillinase had earlier been purified by adsorption on siliceous materials in batches[2] or by fractionation with ammonium sulphate.[3] An improvement in the method of purification was suggested by the application of partition chromatography on Hyflo-Supercel.[4] But the prohibitive cost of the solvents employed precludes the possible use of the technique in large scale production of penicillinase. This led us to investigate on the chromatography of the enzyme in columns as a part of our studies on the production of Antipen.** The present communication deals with methods of purification of the enzyme by adsorption chromatography in columns (Patent pending).

### MATERIALS AND METHODS

#### Penicillinase powder (crude)

Fermention broth of *B. cereus* 5/B NCTC/9946 containing high titre penicillinase[1] was saturated with ammonium sulphate in different stages at suitable pH's and the final precipitate dissolved in a minimum volume of ice cold water. From this, acetone powder was made in the usual way.

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** Trade name of Hindustan Antibiotics Ltd., for injectible penicillinase.

#### Adsorbents

The following adsorbents were employed:

(a) Glass powder : Soft glass powder was further ground in a mortar and sieved through a 150 mesh stainless steel sieve. The glass powder was treated with 0.1*N* HCl, washed free from chlorides and baked at 100° overnight.

(b) Supercel (Johns Manville, U.S.A.).

(c) Glass powder — supercel mixture: An intimate mixture of glass powder —supercel (3:1).

#### Eluants

(a) 0.5 saturated $(NH_4)_2SO_4$ in 0.1*M* $K_2HPO_4$ solution adjusted to pH 9.0 with dilute ammonia.

(b) 4.5 per cent NaCl in 0.1*M* $K_2HPO_4$ adjusted to pH 9.0 with dilute ammonia.

#### Estimations

The enzyme activity was determined by the iodometric method,[1] described earlier. Protein in different fractions of enzyme solution was estimated on a Beckman Spectrophotometer Model DU after Warburg and Christian.[5]

#### Batch operations

For adsorption in batches, crude penicillinase acetone powder (100 mg.) assaying 25,000 units/mg. was dissolved in water (10 ml.) and shaken with glass powder (10 g.) at +5° in a reciprocating shaker (120 strokes/min., length of stroke, 3 cm.)

for 10 min. The solution was then filtered and residual glass powder shaken with three successive portions of eluate (a) for 30, 30 and 15 min. only. After filtration, the filtrates were estimated for enzyme activity.

**Chromatography**

After several trials the following procedure was adopted for chromatography of penicillinase in columns: A slurry of different adsorbents in water was poured into a glass column under mild suction. The solution of the enzyme was then passed through the packed column and elutions were made with different eluants. Fractions were collected and assayed for enzyme activity. In all operations pyrogen-free distilled water was used.

## RESULTS

Table I shows the results of a typical batch operation, when the enzyme was adsorbed on glass powder and subsequently eluted with eluant (a).

It should be noted that although 74 per cent of the enzyme was adsorbed on glass powder in batch process, only 16.9 per cent was eluted, 13.2 per cent of the original enzyme activity being recovered in the first two fractions.

Adsorption in a column packed with glass powder, however, showed that total recovery of the enzyme content was about 80 per cent. Moreover, about 64.0 per cent of enzyme activity was obtained in the most enriched fractions. Fig. 1 represents graphically results of a typical experiment carried out in a column with glass powder as the adsorbent (wet packing) and solution (b) as eluant. It deserves mention that eluant (b) was found to be as efficient as eluant (a); the latter was widely employed by Pollock and co-workers.[2]

Preparation of glass powder for chromatography requires considerable amount of time. Moreover, glass powder fines (unless

TABLE I.

PURIFICATION OF PENICILLINASE BY ADSORPTION ON GLASS POWDER IN BATCHES

| Stage of purification | Vol. (ml.) | Unit (ml.) | Total activity (units) | Recovery Per cent. |
|---|---|---|---|---|
| i) Enzyme solution from acetone dried powder | 10 | 250,000 | 2,500,000 | — |
| ii) Effluent .. .. .. .. .. | 5.5 | 92,000 | 506,000 | 20.2 |
| iii) Wash liquor | | | | |
| (a) .. .. .. .. | 4.0 | 24,000 | 96,000 | 5.8 |
| (b) .. .. .. .. | 5.5 | 9,000 | 49,500 | |
| iv) Eluate | | | | |
| (a) .. .. .. .. | 6 | 48,000 | 288,000 | |
| (b) .. .. .. .. | 6 | 18,000 | 108,000 | 16.9 |
| (c) .. .. .. .. | 5 | 5,140 | 25,700 | |
| Total eluate .. .. .. .. .. | 17 | | 421,700 | |

the whole operation is carried out under water) are likely to be hazardous from the point of view of health of workers. It was, therefore, considered worthwhile to utilize a mixture of glass powder and supercel (3:1) instead of glass powder alone. The recovery of enzyme activity with such a column was 67 per cent, the most enriched fractions containing 51.2 per cent of the total enzyme. The process was faster than that with glass powder alone.

Attempt was next made to use supercel alone for chromatography. Fig. 2 shows the results of a typical experiment carried out with a large batch of crude penicillinase powder. The total recovery of the enzyme was 85-90 per cent. The richer fractions accounted for as much as 75-80 per cent of the initial enzyme activity. Wet packing of the column was found to be more efficient than dry packing.

**Purity of different fractions**

Table II shows the degree of purity attained by different fractions on chromatography through supercel. Specific activity was calculated on the basis of enzyme activity per mg. protein nitrogen. The most enriched fractions were pooled and dialysed against water at +5° till free from Cl.— The dialysed solution was then lyophilized for preparation of Antipen.

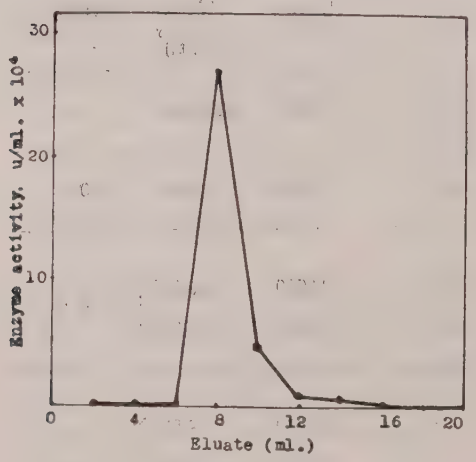


FIG. 1.

Chromatography of crude penicillinase acetone powder on glass (3.0g) Column: 8 cm. x 1.1 cm. (diameter). Charge: 30 mg., at 28,600 u/mg.

## DISCUSSION

The separation of proteins and enzymes by column chromatography has been reviewed.[6,7,8] Moore and Stein[9] as well as Sober and Peterson[10] applied the technique for the purification of proteins. More recently Turba[11] has reviewed recent applications of column chromatography in the field of enzyme purification. Although different adsorbents have been used earlier for purification of penicillinase in batches, no investigation on the chromatography of this enzyme has been reported so far.

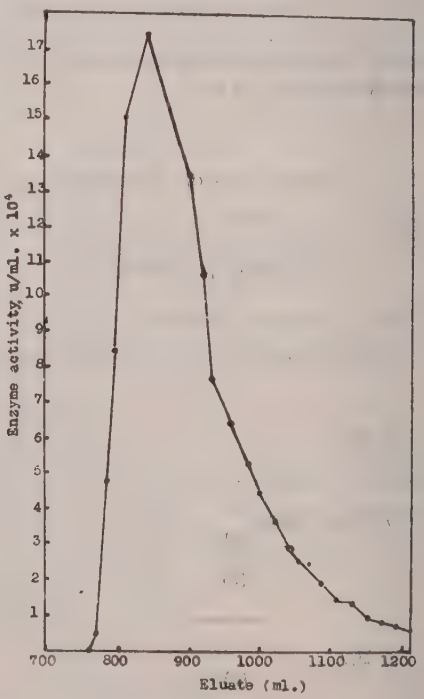


FIG. 2.

Chromatography of crude penicillinase acetone powder on supercel (125 g.) Column: 36.5 cm. x 4.5 cm. (diameter) Charge: 0.955 g. at 33,020 u/mg. Hold-up (water): 360 ml.

TABLE II.

RELATIVE PURITY OF THE ENRICHED FRACTIONS

| Fraction No. | Volume (ml.) | Units/ml. x $10^6$ | Specific activity units/mg. protein N x $10^6$ |
|---|---|---|---|
| 16 | 20 | 3.0 | 0.2 |
| 17 | 22 | 3.8 | 0.328 |
| 18 | 19 | 2.95 | 0.318 |
| 19 | 23.5 | 3.29 | 0.378 |
| 20 | 19.8 | 2.64 | 0.309 |
| 21 | 20.0 | 2.13 | 0.357 |
| | Mean value (fraction 17-21) | | 0.318 |
| | Crystalline penicillinase[3] | | 1.53 |

Penicillinase has recently come to occupy an important place in specific therapy against penicillin allergy. The present investigation was undertaken with the object of finding out suitable methods of purification of crude penicillinase on a large scale for the preparation of penicillinase injectable.

The superiority of the chromatographic method over batch operations is indicated by the fact that it is possible to select more enriched fractions for further processing. With supercel as adsorbent the process is quite rapid and also inexpensive.

From the data on specific activity (Table II) it is evident that the enzyme is enriched fractions is roughly 20 per cent pure as compared crystalline penicillinase which is reported to show specific activity of 1.3 x $10^6$ (mean value).[3] However, the enrichment in enzyme activity per ml. does not run parallel with specific activity per mg. protein nitrogen, presumably because of the presence of contaminant impurities proteinaceous in nature. Nevertheless, the purified enzyme thus obtained in the form of lyophilized powder, passed the British and Indian Pharmacopoeia requirements of all tests for injectables regarding toxicity, pyrogen and histamine-like substances.

The first attempt to crystallize the enzyme by alcohol precipitation according to the method of Pollock *et al.*[3], was successful. Lyophilized enzyme preparation in the dry state has been found to be stable at ordinary temperature. Recent success in the utilization of the technique of counter-current distribution for fractionation of high molecular proteins[12] prompted us to apply this technique in the case of penicillinase. Work in this respect is in progress.

With regards to the relative merits of different adsorbents it should be mentioned that resolution was more efficient when glass powder was used as adsorbent, as is evident from the nature of the curves in Figs. 1 and 2. However, in contrast to supercel, chromatography on glass powder was very slow. Moreover, ready availability of supercel suggested the preference of its use for large scale operations.

This process of column chromatography over supercel has been adopted for the preparation of Antipen.

## SUMMARY

A chromatographic method has been developed for the purification of penici-

llinase. Relative merits of glass powder and supercel as adsorbents are described. The method has been adopted for manufacture of penicillinase injectables for therapy in penicillin allergy.

ACKNOWLEDGEMENT

The authors wish to acknowledge their grateful thanks to Dr. M. R. Pollock, National Institute for Medical Research, London, for supplying the strain of *B. cereus*, and also for checking the potency of Antipen injectable. Thanks are also due to Dr. S. R. Sarvotham for pharmacological tests, and to Mr. B. N. Ganguli, Mr. A. M. Thadathil, and Mr. A. A. Khan for technical assistance.

The authors are also indebted to Dr. M. J. Thirumalachar for his interest in the work.

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# Hamycin, A New Antifungal Antibiotic

## III. MICROBIOLOGICAL AND SPECTROPHOTOMETRIC ASSAY

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THE isolation of an antifungal polyene antibiotic, hamycin, has been recently reported.[1] The antibiotic is produced by *Streptomyces pimprina* Thirum., a new species of *Streptomyces* isolated from soil in Pimpri, India. The mycological aspects and details of isolation, production and characteristics of the antibiotic are under publication.[2,3] Hamycin is active against a large number of fungi, pathogens as well as saprophytes, the yeast-like fungi being especially sensitive. The antibiotic exhibits no appreciable activity against bacteria.

During fermentation, hamycin production is accompanied by simultaneous formation of at least four other antibiotics.[3,4] Hamycin is present exclusively in the mycelium, while the other antibiotics are in the broth. In our attempts to develop suitable assay procedures for estimating hamycin in broth as well as in crystalline preparations, difficulties were encountered due to (*i*) the interference of the other antibiotics present in the broth, (*ii*) high molecular weight of hamycin, and (*iii*) its poor diffusibility in agar.

### ASSAY METHODS

### Microbiological assay

1. *Agar-plate assay*

The zones of inhibition obtained by the conventional agar-plate method of assay were neither clear nor proportional to the concentration of hamycin. This may be due to the poor diffusibility of the compound through agar. In order to overcome this difficulty attempts were made to modify the assay technique by changing the composition of the assay medium and using different test-organisms. However, no significant improvement could be brought about and, therefore, the plate-assay technique could not be standardised.

2. *Turbidimetric assay*

A serial dilution method was standardised using *Candida albicans* as the test-organism. Nine ml. aliquots of the assay medium (yeast extract 0.5%, glucose 1%, glycerine 1%, sodium chloride 1%, and traces of $MgSO_4$ and $KH_2PO_4$, pH 7.0) were distributed in 6″ x 1″ test tubes and autoclaved at 15 lb. for 30 min. Suitable dilutions of the antibiotic, broth or crystalline samples, were added to the test tubes making the final volume in all the tubes to 10 ml. The range of sample dilutions used for assay varied from 1 in 10 million to 1 in 100 million. In general, the activity of hamycin against *C. albicans* was in the range of 1 in 80 million to 1 in 120 million.

A freshly prepared suspension of *C. albicans* in sterile distilled water was used as the inoculum. The tubes were incubated at 28° for 24 hr. At the end of the incubation period turbidimetric readings were taken either visually or with the aid of a photoelectric colorimeter.

*Preparation of sample for assay* : In the case of fermentation broth the anti-

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biotic in the mycelium was extracted using 50 per cent ethanol. Solutions of crystalline preparations were also prepared in 50 per cent ethanol. All dilutions were made in sterile distilled water. The order of dilution was sufficient to nullify any inhibitory effect due to ethanol used in the preparation of the samples.

*Effect of sodium chloride concentration in the medium* : During the course of our investigations it was observed that the concentration of sodium chloride in the assay medium had a marked influence on the sensitivity of the assay. A series of experiments were, therefore, undertaken to evaluate the importance of this observation.

Sodium chloride was added to the medium in graded concentrations from 0 to 2 per cent and the growth of the test organism was followed in the absence of the antibiotic by taking turbidimetric readings at 24, 48 and 72 hr. incubation. From results presented in Fig. 1 it may be seen that as the concentration of sodium chloride in the assay medium increases, there is a small but progressive inhibition of the growth of *C. albicans*. The effect is more pronounced at 24 hr. and is partly overcome after longer incubation period.

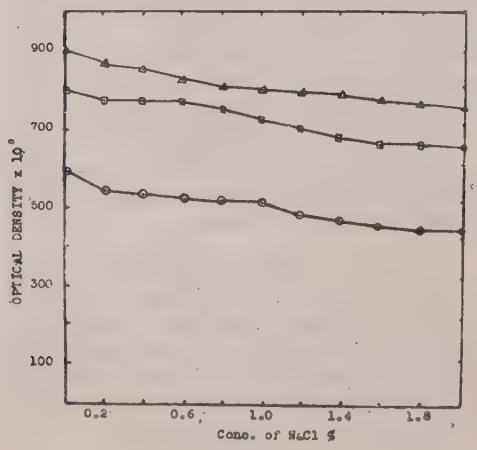


FIG. 1.

Effect of NaCl conc. on growth of *C. albicans*

⊙ 24 hr. ◈ 48 hr. △ 72 hr.

In another experiment sodium chloride was incorporated in the assay medium at 0, 1, and 2 per cent levels and the antibiotic was added at dilutions ranging from 1 in 10 million to 1 in 100 million. The control tubes did not contain any antibiotic. Turbidimetric readings were taken at 24, 48 and 72 hr. The results are presented in Figs. 2, 3 and 4.

Fig. 2 shows the effect of 1 per cent concentration of sodium chloride. This

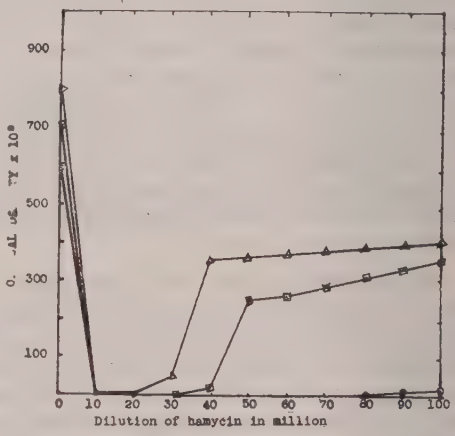


FIG. 2.

Effect of 1 per cent NaCl on growth of *C. albicans* in presence of hamycin

⊙ 24 hr. ◈ 48 hr. △ 72 hr.

represents the assay medium which was used for all the routine turbidimetric estimation of hamycin samples. It is evident that at 24 hr. there is complete inhibition of growth at all the concentrations of the antibiotic. At 48 and 72 hr. almost 40 to 60 per cent of the inhibition is overcome at lower antibiotic concentrations, whereas at dilutions of 1 in 10 million and 1 in 20 million there is complete inhibition.

When the concentration of sodium chloride is increased to 2 per cent (Fig. 3) al-

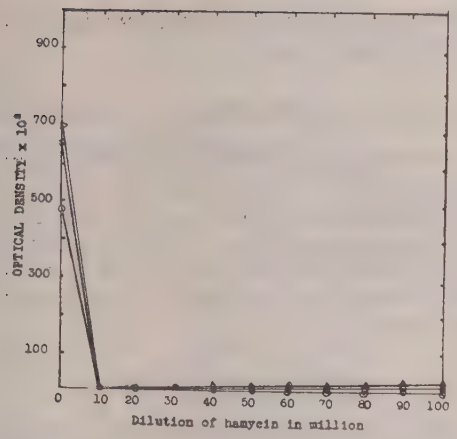


FIG. 3.

Effect of 2 per cent NaCl on growth of *C. albicans* in presence of hamycin

⊙ 24 hr. ◈ 48 hr. △ 72 hr.

most complete inhibition of growth is observed at all concentrations of hamycin and at all periods of incubation. Growth in controls is also reduced to a small extent. In the complete absence of sodium chloride (Fig. 4), the sensitivity of *C. albicans* to hamycin is significally reduced.

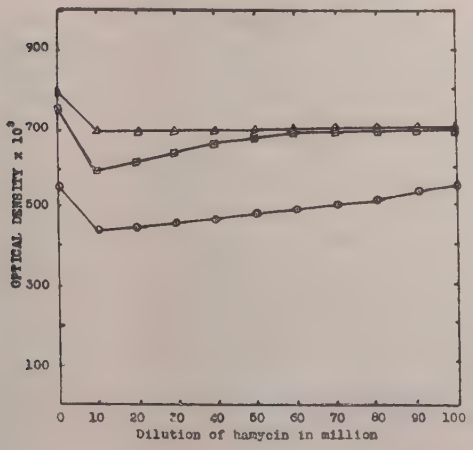


FIG. 4.

Growth of *C. albicans* at varying conc. of hamycin in the absence of NaCl

⊙ 24 hr. ◈ 48 hr. △ 72 hr.

It appears from results in Fig. 1 to 4 that concentration of sodium chloride in the assay medium influences the sensitivity of *C. albicans* in turbidimetric assay for estimating hamycin content in samples. Therefore, all the routine assays were carried out with assay medium containing sodium chloride at 1 per cent level. Although no attempt was made to ascertain the exact mechanism of the effect of sodium chloride on the growth of *C. albicans* in the presence of hamycin, the effect of replacement of sodium chloride with other salts was studied. The comparative effects of sodium chloride, sodium bromide and potassium chloride at 1 per cent level were investigated and the growth of the test-organism was estimated at 24, 48, and 72 hr. of incubation with hamycin at three different concentrations (Fig. 5).

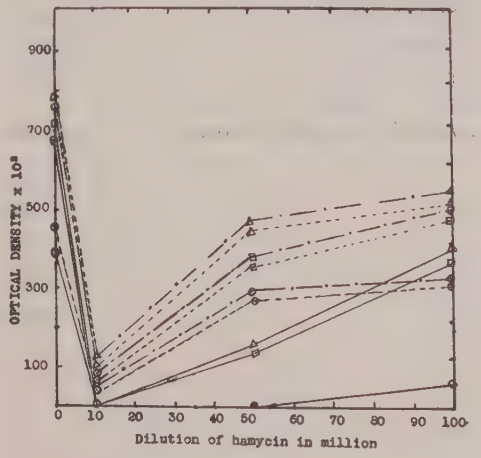


FIG. 5.

Comparative effect of NaCl, NaBr and KBr at 1 per cent level

⊙ 24 hr. ◈ 48 hr. △ 72 hr.

— NaCl - - - NaBr. —·— KBr

It is evident from Fig. 5 that NaCl, NaBr and KCl are well utilized by *C. albicans* for growth, both in the presence and in the absence of hamycin. NaBr and KBr also bring about the inhibi-

tion of growth of *C. albicans* in presence of antibiotic but the degree of inhibition is less than that with NaCl. This difference may be attributed to the fact that the salts were added at 1 per cent level and not at isomolar concentration.

**Spectrophotometric assay**

Although the turbidimetric assay method was entirely satisfactory for accurate estimation of hamycin, it was necessary to develop another sufficiently reliable procedure for ready estimation of the antibiotic in broth samples. A spectrophotometric method, based on the fact that hamycin exhibits a strong ultraviolet absorption at 384 m$\mu$ ($E^{1\%}_{1cm}$ 916) was standardized. The antibiotic, which is present in mycelium, was first extracted either in *n*-butanol or in 50 per cent ethanol and the absorption at 384 m$\mu$ read in a Beckman DU spectrophotometer after appropriate dilutions in the same solvent. A straight line curve for the standard was plotted between concentrations 0 to 10 $\mu$g/ml of hamycin in solvent and contents of the samples were calculated by references to this standard curve.

SUMMARY

Microbiological method for the assay of hamycin is described. The effect of the concentration of NaCl, KBr, and KCl in the assay medium on the sensitivity of the assay was also studied. A spectrophotometric assay method is also briefly described.

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The authors are indebted to Dr. M. J. Thirumalachar for valuable guidance, and to Mr. P. R. Joshi and Mr. N. K. Maladkar for technical assistance.

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# Differential Assay of Phenoxymethylpenicillin (Penicillin V) in Crystalline Samples*

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SEVERAL physical — chemical methods have been reported for the determination of penicillin V in crystalline samples. Any such method has to take into account the facts that (1) in the fermentation of penicillin V, other penicillins are also produced, penicillin G being the principal contaminant due to presence of precursors such as phenylalanine in the complex cornsteep liquor fermentation medium ; (2) crystalline samples of penicillin V isolated from the fermentated liquors are at times contaminated with penicillin G and also the precursor phenoxyacetic acid ; and (3) for routine analysis the method should be fast and fairly accurate.

The paper chromatographic method of Stephens and Grainger[1] for determination of penicillin V in crystalline commercial preparations is tedious for routine analytical work and the results obtained are only 90 to 95 per cent accurate. For the assay of crystalline penicillin V samples contaminated with penicillin G, Goodey *et al.*[2] reported a method based on the inactivation of penicillin G at pH 2 for 3 hr. About 15 to 20 per cent of penicillin V activity is lost at the end of this period so that a correction factor has to be applied for the decomposition of penicillin V. We have investigated the relative stability of potassium salt of penicillin V and penicillin G kept at pH 2 for half an hour. It was found that pure potassium salt of penicillin V loses potency by less than 1.5 per cent (25 units) while practically all penicillin G activity is lost on such treatment[3]. It can, therefore, reasonably be assumed that the penicillin content assayed at the end of this period represents the penicillin V content of the sample containing penicillins V and G.

The U.S. Food and Drug Administration (F.D.A.) describes a spectrophotometric method[4] for the estimation of penicillin V, according to which the characteristic absorption maxima at 275 m$\mu$** of penicillin V in alkaine solution is made use of. The absorption of the sample at 275 m$\mu$ is compared with that of the working standard of penicillin V. Suitable conversion factors are applied if the standard is penicillin V acid and the sample salts. The spectrophotometric method being sensitive is more specific and accurate. Further, penicillin G has no absorption at 275 m$\mu$ and, therefore, does not interfere. But the method is not reliable in the case of preparations containing impurities. The characteristic absorption at 275 m$\mu$ is due to the phenoxymethyl group in the molecule and pure phenoxyacetic acid in alkaline solutions also shows an absorption maxima at 275 m$\mu$ with $E^{1\%}_{1\,cm}$ of 82.90. This high absorption of phenoxyacetic acid interferes with the spectrophotometric determination of penicillin V in preparations

* Forming part of "Studies on Penicillin V" submitted as thesis to the University of Bombay for the degree of Master of Science.

** The British Pharmacopeia[5] prescribes the absorption at 268 m$\mu$ for the same determinations carried out in sodium bicarbonate solution. In our experience the results with the F. D. A. method are better reproducible and agree with the differential assay.

even slightly contaminated with the acid. Table I shows penicillin V content determined by spectrophotometric method in crystals of potassium salt of penicillin V isolated from fermentation broth and also in purified crystals from which phenoxyacetic acid had not been removed. In all the above cases the presence of phenoxyacetic acid was quantitatively established by (1) precipitating pencillin V as calcium salt and isolating phenoxyacetic acid from the mother liquor by acidification, and (2) by estimating phenoxyacetic acid titrimetrically. Phenoxyacetic acid may be estimated in mixtures of penicillin V by the bromination method[6] chloroform extraction,[7] colorimetric method using chromotropic acid[9, 10, 11] or by using the difference in solubility of penicillin V and phenoxyacetic acid in toluene.

The values of penicillin V content estimated by the differential assay described below were checked by spectrophotometric method and were found in close agreement. Solutions of potassium salt of penicillin V of different concentrations were prepared and known amounts of potassium salt of penicillin G added. After carrying out the differential assay iodometrically the solutions were diluted with 0.1*N* sodium hydroxide to a suitable concentration for estimating the penicillin V content spectrophotometrically. The results are presented in Table II.

TABLE I. PENICILLIN V CONTENT IN SAMPLES CONTAMINATED WITH PHENOXYACETIC ACID ESTIMATED ON THE BASIS OF U. V. ABSORPTION AT 275 mμ

| Sample | Potency u/mg. | $E^{1\%}_{1\,cm}$ at 275 mμ | Penicillin V content | Phenoxyacetic acid content |
|---|---|---|---|---|
| A | 1,220 | 32.83 | 122.7 | 3.20 |
| B | 1,400 | 28.80 | 108.5 | 1.00 |
| C | 1,413 | 28.25 | 106.5 | 0.86 |
| D | 1,200 | 34.22 | 129.6 | 3.69 |

TABLE II. PENICILLIN V CONTENT BY DIFFERENTIAL AND SPECTROPHOTOMETRIC ASSAY

| S. No. | Wt. of K Pen. V. mg. | Wt. of K Pen. G. mg. | Pen. G. added % | Total Pen. u/mg. | Potency on keeping soln. at pH 2 for 30 min. u/mg. | Loss in potency % | Pen. V. content by differential assay | $E^{1\%}_{1\,cm}$ at 275 mμ | Pen. V. content by spectrophotometric method |
|---|---|---|---|---|---|---|---|---|---|
| 1. | 76.30 | | | 1,530 | 1,510 | 1.307 | 100.00 | 26.10 | 100 |
| 2. | 72.85 | 1.085 | 1.468 | 1,532 | 1,488 | 2.872* | 98.527 | 25.75 | 98.45 |
| 3. | 73.05 | 2.17 | 2.885 | 1,536 | 1,474 | 4.036* | 97.267 | 25.46 | 97.00 |
| 4. | 73.75 | 3.255 | 4.228 | 1,538 | 1,455 | 5.398* | 96.887 | 24.73 | 95.02 |
| 5. | 70.90 | 4.34 | 5.769 | 1,541 | 1,436 | 6.800* | 94.497 | 24.56 | 94.10 |

*Correction factor was applied on the basis of result in Expt. 1 in which loss of potency of penicillin V was found to be 1.307%.

One of the commercial products of first crystals of potassium salt of penicillin V was assayed for penicillin G and penicillin V content by the differential assay and the composition determined by countercurrent distribution technique.[12] The values obtained by the latter technique are in close agreement with the differential assay (Table III).

TABLE III

DIFFERENTIAL ASSAY AND COUNTERCURRENT DISTRIBUTION OF K PENICILLIN V CRYSTALS (BATCH No. 4126)

| Method | Penicillin G content | Penicillin V content |
|---|---|---|
| Differential assay | 2.84 | 95.15 |
| Countercurrent distribution | 2.90 | 95.04 |

## EXPERIMENTAL PROCEDURE

(1) *Differential assay method for penicillin V.*

An accurately weighed quantity (65-70 mg.) of potassium salt of penicillin V was dissolved in pH 6 phosphate buffer (0.1 *M*) and the volume made upto 50 ml. The assay was carried out iodometrically.

**Blank titre**

To an aliquot (2 ml.) was added 0.01*N* iodine (10 ml.) and titrated against 0.01*N* sodium thiosulphate.

**Test titre No. 1**

To an aliquot (2 ml.) was added 1*N* sodium hydroxide and after 15 min. the solution was acidified with 1.1*N* hydrochloric acid (pH 2-3) ; 0.01*N* iodine (10 ml.) was then added and after keeping for 15 min. titrated against 0.01*N* sodium thiosulphate. The potency of the sample was calculated using the formula :

$$\text{Total penicillin (u/mg.)} = \frac{\text{Blank titre} - \text{test titre} \times 25 \times \text{P. F.}}{\text{Wt. in mg.}}$$

**Test titre No. 2**

To an aliquot (2 ml.) was added 1 or 2 drops of 1.1*N* hydrochloric acid to bring the pH to 2 (for the destruction of penicillin G). After half an hour the solution was neutralized by adding 1*N* sodium hydroxide (1 drop) followed by the addition of 1*N* sodium hydroxide (2 ml.) and the assay carried out as described under test No. 1.

$$\text{Penicillin V (u/mg.)} = \frac{\text{Blank titre} - \text{test titre No. 2} \times 25 \times \text{P. F.}}{\text{Wt. in mg.}}$$

(P. F. = Penicillin factor which was determined using a working standard of potassium salt of penicillin V)[3]

(2) *Procedure for relative stability study of penicillin V and G.*

To 4 different weights of potassium salt of penicillin V (60-70 mg.) were added 1 mg., 1.5 mg., 2.4 mg., and 3 mg., of potassium salt of penicillin G, and dissolved in pH 6 phosphate buffer (0.1*M*) and the solutions made upto 50 ml. Assay was then carried out by differential assay method for the determination of potency of penicillin V.

## SUMMARY

The admixture of penicillin V with penicillin G and the precursor phenoxyacetic acid can lead to false values in the determination of potency and penicillin V content, penicillin G contributing to higher potency and phenoxyacetic acid to increased penicillin V content. In such cases the differential chemical assay would be the method of choice as it estimates the total penicillin content, the penicillin V content, and as there is no interference by the contaminants.

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## THE BOMBAY HOSPITAL JOURNAL RESEARCH POOL FELLOWSHIPS

| FELLOWSHIP | SUBJECT | AWARDED TO |
|---|---|---|
| CARLO ERBA | Treatment of Acute Diarrhoeas in Adults with "MEBIPLEX". | Dr. (Smt.) M. V. Bhansali, B. Y. L. Nair Charitable Hospital, Bombay. |
| CIPLA | Treatment of Cirrhosis of Liver with "CIROZAN". | Dr. J. R. Shah, G. T. Hospital, Bombay. |
| DUMEX | (1) Therapeutic study of "MAXIPEN" (an improved Penicillin) in various infections including sensitivity studies against infecting micro-organisms.<br>(2) Blood level study after varying doses of "MAXIPEN" and its comparison with Penicillin V and other injectable penicillins. | Dr. Banoo D. Curravala, G. T. Hospital, Bombay. |
| HOECHST | Tolbutamide in the treatment of Thromboangitis Obliterans. | Dr. M. J. Joshi, Sassoon Hospitals, Poona-1. |
| SUHRID GEIGY, Fellowship-I. | The clinical aspect of oral diuretic "HYGROTON" in oedematous conditions and estimation of urinary and blood, sodium, potassium and chlorides. | Dr. F. P. Bahadurji, J. J. Hospital, Bombay. |
| SUHRID GEIGY, Fellowship-II. | Hygroton as a hypotensive agent by itself or in combination with other hypotensive agents. | Dr. A. M. Shanbhag, L. T. M. G. Hospital, Bombay. |
| UNICHEM | (1) To evaluate the side reactions of two preparations of Intravenous Iron.<br>(2) To study the hematological response (by assessment of haemoglobin, R. B. C. count, reticulocyte count and serum iron) in iron deficiency anaemia by 3 varieties of iron preparation in tablet form. | Dr. M. S. Narayanan, Government Headquarters Hospital, Tiruchirapalli. |

# Studies on Actinomycin Production in Synthetic Medium

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ACTINOMYCINS form a family of closely related antibiotics elaborated by *Streptomyces* spp. and are known for their action against neoplastic cells. Unfortunately their chemotherapeutic dosages[1] lie within close range of toxicity levels. This led to systematic studies on the preparation of biosynthetic actinomycins culminating in the brilliant report of Schmidt-Kastner[2] and later, of Katz and Goss[3] and others who could produce actinomycins with variations in amino acid composition in the peptide parts of the molecule. The chemistry of actinomycins has been reviewed by Brockmann[4] who recently achieved the total synthesis of this complex molecule.[5] Of significant interest is a report of Chinese workers[6] who claimed the production of a non-toxic actinomycin of therapeutic value. It is, however, desirable that this observation is confirmed in other laboratories as well.

Thirumalachar and Ghosh,[7] isolated from the culture filtrate of *Streptomyces chrysomallus* Linden. (=*S. griseus* of some authors) (strain No. 1710), an antibiotic which was identified as actinomycin. For the present study a synthetic medium was selected as against natural medium used earlier[7] in order to obtain sufficient data that will enable further investigations on the biosynthesis of new actinomycins with suitable amino-acid precursors. Emphasis has here been laid on the metabolic aspects of the fermentation in shake culture flasks with the idea that the pattern revealed may be useful for a practical fermentation on a large scale.

## MATERIALS AND METHODS

*Streptomyces chrysomallus* Linden. (strain No. 1710)* was used for the production of actinomycin.

### Medium

The medium used was composed of the following ingredients (in g.) : (A) Glutamic acid 2.0 ; $NaNO_3$, 1.0 ; $MgSO_4 7H_2O$, 0.025 ; $ZnSO_4 7H_2O$, 0.025 ; $FeSO_4 7H_2O$, 0.025 ; $CaCl_2$, 0.025 ; $H_2O$ upto 750 ml. ; pH 7.2.

The above medium (75 ml.) was dispensed in 500 ml. Erlenmeyer flasks and autoclaved.

(B) Lactose hydrolysate : For preparation of lactose hydrolysate, lactose (80 g.) was dissolved in water (900 ml.) to which was added conc. HCl (10 ml.). The solution was autoclaved for 30 min. at 15 lb. pressure for hydrolysis. The pH was then adjusted to 7.0 with NaOH and the total volume was made upto 1,000 ml. The lactose hydrolysate was distributed in test tubes, each containing 25 ml. of the solution and autoclaved.

Prior to inoculation, lactose hydrolysate (25 ml.) (B) was added to medium (75 ml.) (A) aseptic conditions.

### Fermentation

(a) *Preparation of seed cultures.* The cultures were prepared by inoculating spores from potato-agar slopes into the medium (100 ml.) having the above

* Kindly provided by Dr. M. J. Thirumalachar, Superintendent Research, Hindustan Antibiotics Ltd., Pimpri.

composition and allowing them to grow on a rotary shaker (240 r.p.m., 2″ throw) for 3-4 days at 25-26°.

(b) *Fermentation:* For fermentation, the micro-organisms was grown at 25-26″ for 7-8 days in 500 ml. Erlenmeyer flasks previously seeded with 1/10th volume of water washed seed cultures.

**Estimations**

Actinomycin was estimated at 440 mμ on a Bausch and Lomb Colorimeter (Spectronic 20). For bioassay, the routine agar plate assay method was employed with *B. subtilis* as the test organism.

The pH was estimated with a Beckman pH-meter.

Nitrogen was estimated by the microkjeldahl method as modified by Ma and Zuazaga.[8]

The growth of the micro-organism at different time periods was followed by filtering the growth suspension through tared filter papers (Whatman No. 1) washing with water and drying them at 100° overnight.

A qualitative study of the utilization of lactose hydrolysate was made by paper chromatography with butanol-acetic acid-water (4:1:5) as the developing solvent and benzidine trichloracetic acid as the spray reagent.

For the resolution of the actinomycins by paper chromatography the solvent mixture cyclohexane, methanol, benzene, propylene, glycol (1:1:1:1)[10] was tried for want of *n*-dibutyl ether.

For acid hydrolysis of actinomycin the antibiotic (3.5 mg.) and conc. HCl (0.1 ml.) were sealed in a pyrex tube and kept at 120° for 32 hr. The excess of HCl was removed from the hydrolysate by evaporation over a water bath.

The amino-acid composition of actinomycin was determined by paper chromatography, both one dimensional and two dimensional, in the usual way.

Counter current distribution studies were carried out in a 50 tube semi-automatic Craig's apparatus (Quickfit) using $CCl_4$ and 4.5 per cent Na-β-naphthalene sulphonate as solvent phases.[9]

## RESULTS

### (a) Fermentation

Fig. 1 shows the variation of pH, the antibiotic titre and the growth of the micro-organism with time. It may be noted that growth was maximum at the end of 144 hr. followed by a decline, possibly due to autolysis, with a sharp rise in the pH. The

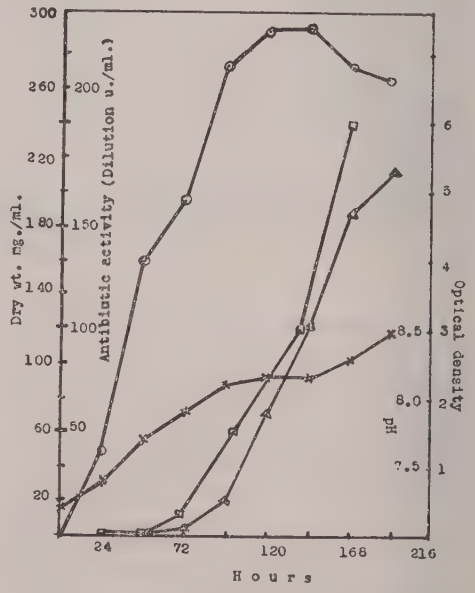


FIG. 1.

Variation of pH, titre and growth of *Streptomyces* sp. (strain No. 1710) with time

⊙——⊙ Mycelial dry wt. in mg./100 ml. of medium
×——× pH
△——△ Actinomycin titre by colorimetry
□——□ Actinomycin titre by bioassay

antibiotic titre in the filtered broth was found to attain maximum value between 168 hr. and 192 hr. as measured both by colorimetry and bioassay.

Fig. 2 shows the utilization of carbon and nitrogen. Marked utilization of hydrolysed lactose took place at the end of 72 hr. although a steady fall in sugar-content was noted right from the start of the fermentation till 144 hr. when 20.0 per cent sugar was left unutilized. From Fig. 3 it is evident that although glucose and galactose were metabolised at the end of 144 hr., part of unhydrolysed lactose (15-20 per cent) was left unassimilated till the end.

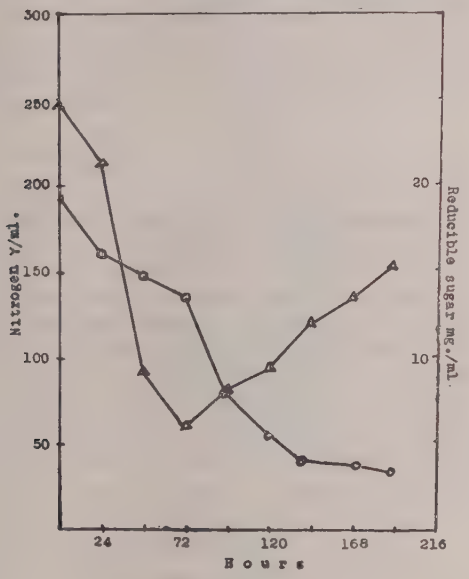


FIG. 2.

Utilization of reducible sugar and nitrogen source by *Streptomyces* sp. (strain No. 1710) during actinomycin production.

○——○ Reducible sugar utilization

×——× Nitrogen utilization

With regard to $N_2$-utilization, the mapping became rather complicated owing to the fact that actinomycin production started from 72 hr. onwards. It can be concluded, however, that about 75-80 per cent of the $N_2$-source was metabolised before the expiry of 72 hr. An attempt was made to estimate glutamic acid by paper chromatography but this had to be abandoned due to the difficulty already mentioned.

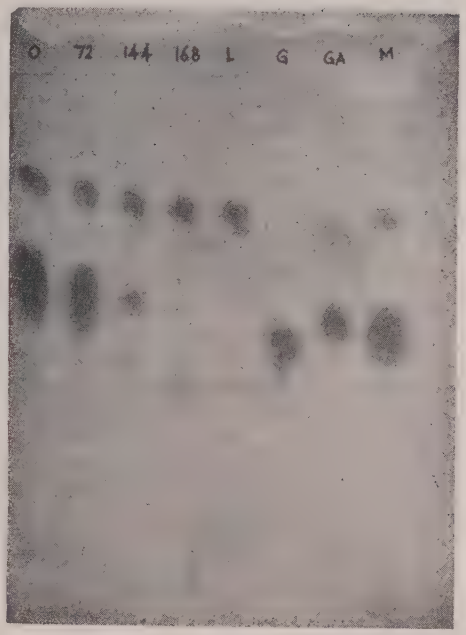


FIG. 3.

Utilization of hydrolysed lactose by *Streptomyces* sp. (strain No. 1710)

L=lactose; G=glucose; GA=galactose; M=mixture

### (b) Extraction and characterization of actinomycin

Culture filtrate (81.) assaying 225-50 u/ml. (dilution units) was processed for extraction first with *n*-butanol followed by evaporation under vacuum. The crude residue was refluxed with benzene and soluble part evaporated in a shallow porcelain dish on water bath to give 0.7-0.8 gm. of crude actinomycin assaying 2,275 u/mg.

The resolution of the different components was not satisfactory by paper chromatography with the solvent used.

Chromatography over silica gel[10] was also not very efficient for, although three distinct bands were obtained, they were contaminated with a yellowish compound trailing all through so that isolation of pure components became difficult.

Fig. 4 shows the counter current distribution pattern of crude actinomycin. For

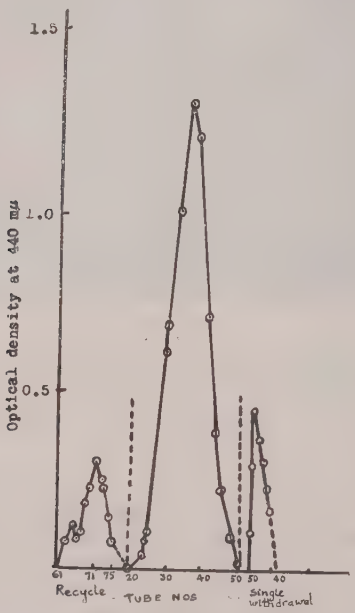


FIG. 4.
Countercurrent distribution of crude actinomycin.

want of *n*-dibutyl ether, the solvent phases proposed by Zepf[9] were employed. It was observed that three distinct bands were visible on a 75-tube transfer in a 50-tube apparatus, the last 25 transfers being made by recycling. But on continuation of transfers upto 95, the two faster moving bands merged. So, in the next operation, after the first 50 transfers were carried out by fundamental procedure, the band migrating first was withdrawn (10 transfers) followed by recycling. In this way the number of transfers was reduced.

The fractions obtained were pooled together separately and pure components isolated according to the method of extraction already outlined. The percentages of fractions recovered and the theoretical percentages calculated from distribution curves were as follows :

| *Fractions* | *Percentage* *Recovered* | *Theoretical* |
|---|---|---|
| A | 75 | 78.0 |
| B | 10 | 11.4 |
| C | 6 | 11.3 |

### (c) Determination of the amino-acid composition of actinomycins

Sarcosine, valine, threonine, *iso*-leucine, proline, and N-methyl valine appeared to be present in the acid hydrolysate of crude actinomycin as determined by paper chromatography. The presence of N-methyl-valine was corroborated by spot test with *p*-nitrobenzoylchloride in the presence of pyridine.[11]

## DISCUSSION

Actinomycins have been exhaustively studied since 1940 when Waksman and Woodruff[12] first reported the so-called actinomycin A. The metabolic pattern in complex media has also received considerable attention.

It is known that different strains of *Streptomyces* species elaborate not only different actinomycins but more often the percentage compositions of the different fractions belonging to one group also vary. Katz *et al.*[13,14,15] employed synthetic media in their metabolic studies on actinomycins. The medium selected for the present investigation is essentially a modification of theirs with some alterations suited to our purpose. Hydrolysed lactose was used instead of galactose alone because of the prohibitive cost of the latter. Figs. 1 and 2 show that the metabolic pattern followed by strain 1710 and is in close agreement with that reported by Katz *et al.*[13]

They noted that about 35 per cent of galactose remained unutilized while in the present case part of unhydrolysed lactose was not assimilated. It was earlier found that lactose by itself could not support the growth of the organism for production of actinomycin which is in conformity with Katz's observation. The utilization of hydrolysed lactose without marked effect on the antibiotic titre suggests the possibility of its use as carbon source for large scale production purposes.

To the best of our knowledge the metabolism of nitrogen source has not been studied in synthetic media possibly because of the difficulties inherent in the nature of actinomycin. It has already been pointed out that the production of the antibiotic commenced between 72 and 96 hr. Actinomycin contains two peptide chains and this renders it extremely difficult to study the utilization of organic nitrogen sources. Results of investigations on the utilization of different amino-acids and peptides, sulphur containing amino-acids and peptides in particular, will be reported later.

The counter current distribution pattern (Fig. 4) demonstrates the presence of three components in the crude mixture. The possibility is recognized that with larger number of transfers and different solvent pairs different distribution patterns may be obtained.

With regard to characterisation of the crude antibiotic it has been noted that the peptide part contains threonine, valine, N-methyl valine, sarcosine, proline and isoleucine. The antibiotic could be classified only after the quantitative aspects of the amino-acid contents of the different fractions are completed. Work is in progress in this respect. The presence of *iso*-elucine itself does not indicate that the antibiotic belongs to actinomycin C group for, there are biosynthetic actinomycins other than actinomycin C, which contain *iso*-leucine.[5] Fortunately, in a recent symposium (1960) a certain degree of systematization of this broad and complex antibiotic group has been made. It is expected that the total synthesis of actinomycins achieved by Brockmann will go a long way in clarifying the complexities of characterisation of actinomycin group.

SUMMARY

An investigation on the production of actinomycin by a strain of *Streptomyces chrysomallus* showed that hydrolysed lactose can be successfully utilized as a cheap carbon source for practical fermentation in synthetic medium. The metabolic pattern followed by the organism during production of the antibiotic was mapped. The relative percentages of the components synthesised in crude actinomycin were determined.

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# Oxidation of Acetate, Pyruvate and TCA-Cycle Acids by *Penicillium Chrysogenum* Spores

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ATTEMPTS to demonstrate TCA-cycle enzymes in spores of micro-organisms have been made only recently.[1] Spores are characterized by low rate of respiration, high resistance to heat, impermeability towards substrates, and hence the enzymatic studies have been mostly carried out with cell-free extracts. In many micro-organisms it has been found that enzyme systems abundantly demonstrable in vegetative cells could not be detected in spores tested concomitantly, but are developed during the course of germination.[2-5] Though extensive investigations have been carried out with respect to bacterial spores,[6] very little is known about the metabolism of fungal spores.

Oxidation of all TCA-cycle intermediates by intact vegetative cells of *Penicillium chrysogenum* at low pH, has recently been demonstrated using mycelial samples from commercial penicillin fermentations.[7] The present communication deals with the oxidation of acetate and TCA-cycle acids by spores of *Penicillium chrysogenum*.

## MATERIALS AND METHODS

Spores of *Penicillium chrysogenum* HA-9 (a derivative of a Russian strain) were grown on barley grains containing honey-peptone mixture in a 500 ml. Erlenmeyer flask at 24° for 6-8 days. After spore growth the barley flask could be preserved for about a fortnight in cold room at +5° without loss of respiratory activity of the spores. When required, spores from one flask (100 g. barley grains) were suspended in distilled water (100 ml.) and gently shaken to dislodge the spores from barley grains, the suspension carefully decanted and centrifuged. The spores formed a distinct layer over a white layer of barley starch in the centrifuge tube. The spore layer was carefully removed without disturbing the lower starch layer and was re-suspended in distilled water. The spore suspension was recentrifuged and the process of separating the top spore layer was repeated until the spores were free from adhering starch granules as determined by microscopic examination after staining with iodine. The spores were finally suspended in a suitable volume of buffer and aliquots were used for manometric study as well as for dry weight determinations. Oxygen uptake was measured by the conventional manometric technique,[8] using a refrigerated circular Warburg apparatus (American Instruments Co.). Dry weights were determined by evaporating a suitable aliquot of spore suspension in buffer (the same that is used for manometric study) to dryness at 100° for 24 hr., corrections being made for the buffer.

Oxidation of the following compounds was studied :

1. Na pyruvate (Schwarz chemicals)
2. Na acetate, neutral (E. Merck)
3. Na citrate (E. Merck)
4. Succinic acid (E. Merck)
5. *cis*-Aconitic acid†

* Present address: Central Drugs Laboratory, Kyd Street, Calcutta.

† Kindly provided by Dr. H. A. Hardy, University of Wisconsin, Madison, Wis.

6. Fumaric acid (Eastman)
7. Malic acid (Eastman)
8. ∝-Ketoglutaric acid (Mann Research Laboratories)
9. Oxaloacetic acid (Mann Research Laboratories).

The substrates were used in the form of 0.1*M* solutions adjusted to pH 7.0. The system consisted of spore suspension in buffer (1.0 ml.) and buffer (1.5 ml.) in the main compartment, substrate (0.5 ml.) in side arm, and 5*N* KOH (0.2 ml.) in the centre cup. Oxygen uptake rates were measured for a period of 5 hr. after tipping the substrate, temperature of the bath being maintained at 24° throughout the study.

## RESULTS AND DISCUSSIONS

It will be seen from Fig. 1 that only acetate was rapidly oxidized by *P. chryso-genum* spores, all the rest of the acids except oxaloacetic acid did not show any increase of oxygen uptake over the endogenous even after a lag period of five hours. Stimulation by oxaloacetate, however, was very little compared to that by acetate. Similar results were also obtained with intact vegetative cells of the mould at pH 7.0.[7] Failure of these TCA-cycle acids to stimulate respiration of intact cells of other microorganisms has also been reported.[9, 10] However, partially ruptured cells[11] and pre-starved cells[7] were shown to oxidize these acids at pH 7.0. With *P. chrysogenum* spores, attempts to break the cells by blenderization or grinding with glass powder, sand or alumina proved unsuccessful. Oxidation of these acids was, therefore, studied with intact spores at pH 2.6 at which pH oxida-

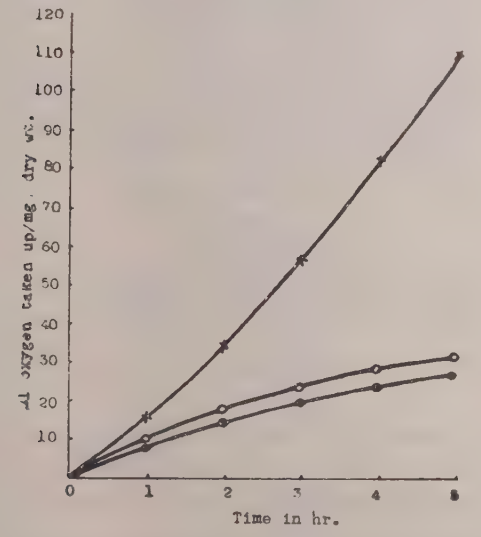


FIG. 1.

Oxidation of TCA—cycle intermediates by *P. chrysogenum* (HA-9) spores at pH 7.0, 24

●——● Endogenous, pyruvate, citrate, *cis*-aconitate, a-ketoglutarate, succinate, fumarate and malate
○——○ oxaloacetate
×——× acetate
Substrate conc. 50 µM per flask
Buffer: O. 1*M* phosphate buffer pH 7.0

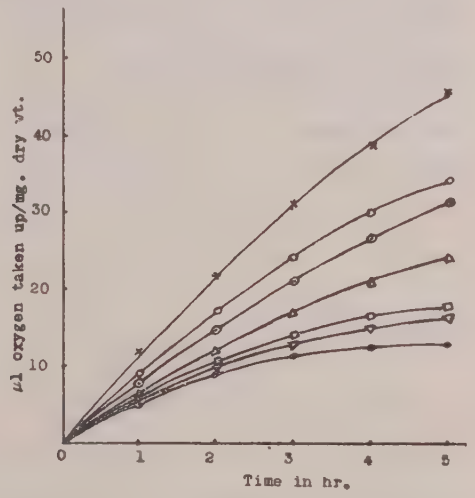


FIG. 2.

Oxidation of TCA-cycle intermediates by *P. chrysogenum* (HA-9) spores at pH 2.6, 24°

●——● Endogenous
▽——▽ *cis*-Aconitate, a-ketoglutarate, fumarate and malate
□——□ Citrate
△——△ Succinate
⊙——⊙ Oxaloacetate
○——○ Pyruvate
×——× Acetate
Substrate conc.: 50 µM per flask
Buffer: 0.1*M* phthalate-HCl buffer pH 2.6

tion of TCA-cycle acids could be easily demonstrated in intact mycelial cells of *P. chrysogenum*.[7] Results of a typical experiment are shown in Fig. 2. Acetate, pyruvate, oxaloacetate and succinate showed marked stimulation while citrate, *cis*-aconitate, α-ketoglutarate, fumarate and malate were slowly oxidized by the spores. The existence of TCA-cycle operation in the spores is thus indicated. It is interesting to note that in intact hyphal cells acetate was found to inhibit endogenous respiration at pH 2.6 ; pyruvate showed stimulation of oxygen uptake only at neutral pH but was without any effect at low pH ; all the rest of the acids markedly stimulated oxygen uptake at pH 2.6.[7] This difference in response to substrates between spores and hyphal cells may be partly due to the nature of the cell wall and partly due to the lack of adequate synthesis of necessary enzymes in spores.

**Pathway of oxidation of acetate**

Oxidation of acetate was studied in presence of arsenite and malonate at pH 7.0 and pH 2.6 (Table I). Acetate oxidation was partially inhibited by arsenite at pH 7.0 only. Endogenous respiration remained unaffected in presence of these inhibitors. Since at the highly acidic pH of 2.6 the cell wall permeability may be considered to be favourable to the entry of substrates, the above results indicate that glyoxylate bypass is perhaps the major pathway of acetate oxidation in the spores of this mould. This is further substantiated by the following experiment on complete oxidation of acetate. Two flasks were run, one without acetate *i.e.*, only endogenous (a), and the other with 5 μM acetate (b), containing same amount of spore suspension. As long as acetate was oxidized, flask (b) showed higher oxygen uptake values. After complete oxidation of acetate, the rates of respiration in both the flasks (a) and (b) became equal. The difference in total oxygen uptake in these two flasks could be assumed to be due to complete oxidation of 5 μM acetate. From results presented in Table II it will be seen that for complete oxidation of 5 μM of acetate, 125 μl of oxygen was used up, *i.e.*, 25 μl oxygen per μM of acetate.

TABLE I. EFFECT OF INHIBITORS ON OXIDATION OF ACETATE BY *P. chrysogenum* (HA-9) SPORES AT 24°

| Substrate | μl oxygen/mg. dry wt. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | pH 7.0 | | | | | pH 2.6 | | | | |
| | HOURS | | | | | HOURS | | | | |
| | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 |
| Endogenous .. .. .. | 8.5 | 15.4 | 20.1 | 23.7 | 26.1 | 5.0 | 8.7 | 11.4 | 13.4 | 14.7 |
| End. + Arsenite .. .. .. | 8.1 | 14.7 | 19.2 | 22.8 | 25.2 | 5.3 | 9.5 | 12.4 | 14.6 | 16.1 |
| End. + Malonate .. .. .. | 8.8 | 15.6 | 20.4 | 23.9 | 26.5 | 5.3 | 8.9 | 11.6 | 13.5 | 15.0 |
| Acetate .. .. .. .. | 17.7 | 36.8 | 61.4 | 86.3 | 111.8 | 16.5 | 32.2 | 45.5 | 58.5 | 69.9 |
| Acetate + Arsenite .. .. | 13.0 | 25.0 | 40.6 | 56.4 | 72.6 | 16.8 | 31.8 | 44·6 | 57.4 | 68.8 |
| Acetate + Malonate .. .. | 17.7 | 36.8 | 61.4 | 86.0 | 111.5 | 16.8 | 32.6 | 46.7 | 59.5 | 71.1 |

1. Concentration of acetate was 50 μM/flask. 2. Final concentration of inhibitor was 0.01M.
3. Buffers : { 0·1*M* phosphate buffer pH 7.0 / 0.1*M* phthalate-Hcl. pH 2.6.

TABLE II. OXIDATION OF ACETATE BY *P. Chrysogenum* SPORES (HA-9) AT pH 7.0, 24°

| Substrate | $\mu$l oxygen uptake | | | | | | Total oxygen consumption in 5 hr. |
|---|---|---|---|---|---|---|---|
| | 1st hr. | 2nd hr. | 3rd hr. | 4th hr. | 5th hr. | 6th hr. | |
| (a) Endogenous | 29.3 | 24.6 | 21.6 | 18.5 | 18.5 | 12.3 | 112.5 $\mu$l |
| (b) Acetate (5 $\mu$M) | 60.5 | 63.5 | 55.9 | 36.3 | 21.2 | 12.1 | 237.4 $\mu$l |
| | | | | | | Difference | 124.9 $\mu$l |

If acetate is completely oxidized via TCA cycle, then according to equation (1), one $\mu$M of acetate would consume

$$\text{Acetate} + 2O_2 \rightarrow 2CO_2 + 2H_2O \quad \text{....(1) (for TCA-cycle)}$$

224 $\mu$l of oxygen for complete oxidation. But if it is oxidized via glyoxylate bypass, then according to equation (2) one $\mu$M

$$2\ \text{Acetate} + \tfrac{1}{2}O_2 \rightarrow \text{succinate} \quad \text{....(2)}$$
(for glyoxylate bypass)[12]

of acetate would consume only 28 $\mu$l of oxygen. Thus, from the observed value of 25 $\mu$l of oxygen utilization per $\mu$M of complete oxidation of acetate (Table II), it may be concluded that acetate is rapidly oxidized via glyoxylate bypass in spores as a readily assimilable carbon source. Presence of *iso*-citric lyase enzyme characteristic of glyoxylate bypass has actually been shown by Olson[13] in cell free extracts of *P. chrysogenum* grown in acetate.

## SUMMARY

Existence of the Tricarboxylic Acid Cycle operating in the intact spores of *P. chrysogenum* was postulated from manometric data on stimulation of oxygen uptake in presence of TCA-cycle intermediates at pH 2.6.

Acetate appeared to be rapidly oxidized by spores via glyoxylate cycle.

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## SHORT NOTES

# N, N′-Dialkylethylenediamine Dipenicillin V Salts

**A PRELIMINARY NOTE**

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SOME undesirable features of penicillin G (benzylpenicillin) in therapeutic use are its acid lability leading to its rapid inactivation in the stomach, and high solubility causing a rapid secretion from the system. Some of the amine salts of penicillin G have been found to be more satisfactory and effective therapeutic preparations.[1] Of particular interest are the ethylenediamine salts of penicillin G such as *N,N′*-dibenzylethylenediamine dipenicillin G (Benzathine or Bicillin) which has proved to be clinically useful.[2] Recently, favourable results have also been reported with *N,N′*-di-*n*-octylethylenediamine salts of penicillin G[3].

Since phenoxymethylpenicillin (penicillin V) is acid stable it could be expected to yield amine salts with greater repository action than the corresponding penicillin G salts. We have, therefore, prepared several penicillin V salts with alkyl substituted ethylenediamines with a view to study their biological properties. In the present preliminary communication, some of their physical and chemical properties are reported (Table I).

The preparation and properties of the intermediates *N,N′*-dialkyl, *N,N′*-di-*p*-toluene sulphonylethylenediamines as well as the bases *N,N′*-dialkylethylenediamines have been described earlier by Vyas and Dhopate[3] who used Hinsberg[4] procedure for their preparation. The same procedure was adopted for the preparation of the bases and the salts in the present experiments. As expected the potencies of the compounds and also their solubilities decreased with increasing molecular weights.

TABLE I. N,N′—DIALKYLETHYLENEDIAMINE DIPENICILLIN V.

$\left.\begin{matrix} CH_2\text{-}NH\text{-}R \\ CH_2\text{-}NH\text{-}R \end{matrix}\right\}$ 2 Penicillin V.

| S. No. | R | M. P. | Yield % | Potency[5, 6] u/mg. | | Solubility in $H_2O$/ml. | | Taste | pH of aq. suspension | Moisture[7] % | Formula | N-Analysis % | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Found | Calc. | Units | mg. | | | | | Found | Calc. |
| 1 | *n*-Butyl | 91-93° | 69 | 1,302 | 1,308 | 4,950 | 3.8019 | Bitter | 5.2 | 4.12 | $C_{42}H_{60}O_{10}N_6S_2 .2H_2O$ | 8.8 | 9.2 |
| 2 | *n*-Amyl | 84-86° | 83 | 1,209 | 1,270 | 3.509 | 2.902 | Bitter | 6.2 | 4.36 | $C_{44}H_{64}O_{10}N_6S_2 .2H_2O$ | 8.6 | 8.9 |
| 3 | *iso*-Amyl | 90-91° | 94 | 1,217 | 1,270 | 2,616 | 2.149 | Bitter | 6.4 | 3.80 | $C_{44}H_{64}O_{10}N_6S_2 .2H_2O$ | 8.9 | 8.9 |
| 4 | *n*-Hexyl | 69-70° | 86 | 1,186 | 1,288 | 1,787 | 1.507 | Bitter | 5.1 | 7.21 | $C_{46}H_{68}O_{10}N_6S_2 .4H_2O$ | 8.3 | 8.4 |
| 5 | *n*-Heptyl | 80-81° | 90 | 1,144 | 1,155 | 638 | 0.559 | Slightly bitter | 5.4 | 7.58 | $C_{48}H_{72}O_{10}N_6S_2 .4H_2O$ | 7.5 | 8.1 |

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# Further Studies on Actinomycin Complex Produced by A *Streptomyces* Species

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IN a preliminary note[1] the production of an antibiotic substance belonging to the group of actinomycins in soybean-glucose medium by a strain of *Streptomyces* sp. classified under *S. chrysomallus* Linden. (*S. griseus* of some authors) was reported. The crude actinomycin, isolated from the culture filtrate of a 8 day growth in shake flasks at 28°, was fractionated by chromatography over Brockmann's alumina and eluted with petrol-ether (60-80°), benzene and benzene-ethylacetate mixture (3:1) in succession. Three main fractions were collected and actinomycins were crystallized from acetone-petroleum ether (60-80°). On a 50-stage countercurrent distribution between benzene-petrol ether (1:4) and 30 per cent urea solution[2] the fraction C further resolved into two distinct components. On the basis of ultraviolet analysis, the compound was finally established as belonging to actinomycin group.

Table I summarises properties of different fractions along with their antimicrobial spectra.

The crude actinomycin complex called Rossimycin A before identification showed anticarcinogenic activity against Ridgeway osteogenic sarcoma comparable to that of actinomycin D. However, even 0.05 mg./kg. showed adverse effect on weight gain in test animals.

TABLE I. PROPERTIES OF ACTINOMYCIN FRACTIONS

| Fraction* | M. P. | Solubility | Minimum inhibitory concentration, μg./ml. | | | | |
|---|---|---|---|---|---|---|---|
| | | | *S. aureus* | *Sarcina lutea* | *E. coli* | *Salmonella paratyphi* | *Mycobacterium phlei* |
| A | 180 (dec.) | Insoluable in benezene but soluble in 10% alcohol | — | <2 | — | — | — |
| B | 218-20 (dec.) | Soluble in benzene and 10% alcohol | — | — | — | — | — |
| C | 240-43 (dec.) | Soluble in benzene and 10% alcohol | <0.5 | <0.5 | — | — | >0.5< |

— indicates no activity.
* Actinomycin fractions were dissolved in 10% alcohol and proper dilutions were made with distilled water.

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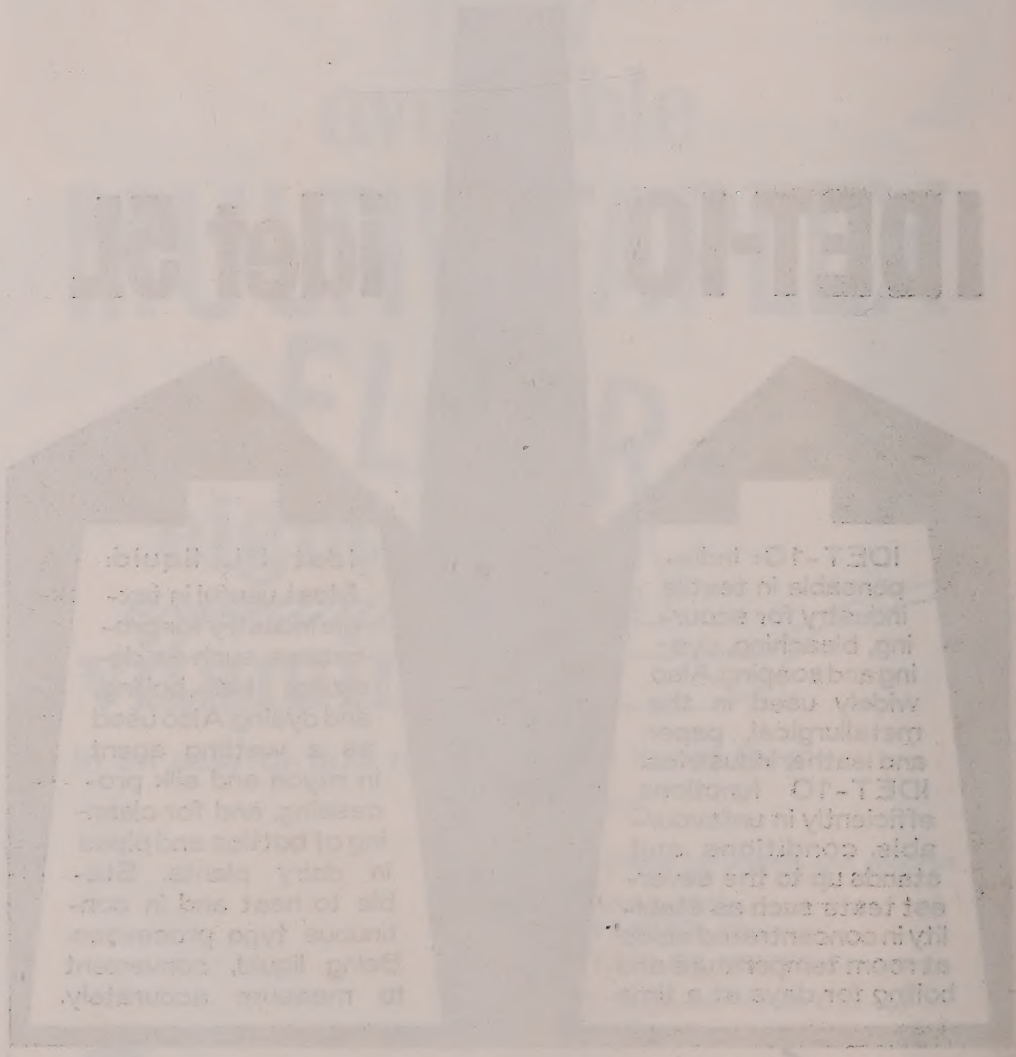

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